INTERACTIONS BETWEEN ENDOGENOUS PRIONS, CHAPERONES AND POLY-GLUTAMINE PROTEINS IN THE YEAST MODEL

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CHAPERONES AND POLY-GLUTAMINE PROTEINS
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I dedicate this thesis to my parents, Mr Pramod and Mrs Deepa Bapat.

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LIST OF SYMBOLS AND ABBREVIATIONS

aa	amino acid
AD	Alzheimer's disease
Amp	Ampicillin
ALS	Amyotrophic Lateral Sclerosis
ATP	adenosine triphosphate
bp	base pair
BSE	Bovine spongiform encephalopathy
CBP	CREB binding protein
CJD	Creutzfeldt-Jacob disease
CSK	cytoskeleton
CUP1	indicates the strong $CuSO_4$-inducible *CUP1* promoter
$CuSO_4$	copper sulfate
DLB	Dementia with lewy bodies
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Gal	galactose
GAL	indicates the strong galactose-inducible *GAL1,10* promoter
GFP	Green Fluorescent protein
GSS	Gerstmann-Straussler-Scheinker disease
GuHCl	guanidine hydrochloride
HD	Huntington's disease
Hsp(s)	Heat shock protein(s)
Htt	Huntingtin protein
kb	kilobase
kDa	kilodalton
Lat A	latrunculin A
LB	Luria broth
LBs	Lewy bodies
MJD	Machado-Joseph Disease
MgCl	magnesium chloride

MSA	Multiple System Atrophy
NaCl	sodium chloride
NBD	nucleotide binding domain
NFTs	neurofibrillary tangles
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PFD	prion forming domain
PMSF	phenylmethylsulfonyl fluoride
poly-Q	poly glutamine
PFD	prion forming domain
Q25	normal polyglutamine stretch of 25 glutamine residues in exon 1 of huntingtin
Q103	expanded polyglutamine stretch of 103 glutamine residues in exon 1 of huntingtin
Raf	raffinose
RNA	ribonucleic acid
SCA	Spinocerebellar ataxia
SDS	sodium dodecyl sulfate
Sp	indicates the endogenous S. cerevisiae promoter for a given ORF
SPs	senile plaques
TBP	Tata binding protein
TE	10 mM Tris-HCl, 1 mM EDTA, pH 7.4
Ub	Ubiquitin
WT	wild-type
YPD	1% yeast extract, 2% bacto peptone, 2% dextrose
YPG	1% yeast extract, 2% bacto peptone, 2% glycerol

SUMMARY

Poly-Q expanded exon 1 of huntingtin (Q103) fused to GFP is toxic to yeast cells containing endogenous yeast prions, [PIN$^+$] ([RNQ$^+$]) and/or [PSI$^+$], which presumably serve as aggregation nuclei. Propagation of yeast prions is modulated by the chaperones of Hsp100/70/40 complex. While some chaperones were reported to influence poly-Q aggregation in yeast, it was not clear whether they do it directly or via affecting yeast prions. Our data show that while dominant negative Hsp104 mutants antagonize poly-Q aggregation and toxicity by eliminating endogenous yeast prions, some mutant alleles of Hsp104 decreases size and ameliorate toxicity of poly-Q aggregates without affecting prion propagation. Elevated levels of the yeast Hsp40 proteins, Ydj1 and Sis1, exhibit opposite effects on poly-Q aggregation and toxicity without influencing prion propagation. Among the yeast Hsp70s, only overproduction of Ssa4 antagonized poly-Q toxicity. We have also isolated dominant Anti-poly-Q-toxicity (*AQT*) mutants counteracting poly-Q toxicity only in the absence of the major ubiquitin-conjugating enzyme Ubc4. Prion forming potential of other Q-rich proteins and influence of Q and P-rich regions on prion propagation were also studied. Our data connects poly-Q aggregation and toxicity to the stress defense pathway in yeast. As many stress-defense proteins are conserved between yeast and mammals, our data shed light on possible mechanisms modulating poly-Q aggregation and toxicity in mammalian cells.

CHAPTER 1

INTRODUCTION

Mammalian neurodegenerative disorders

Role of protein aggregation in mammalian neurodegenerative disorders

Neurodegenerative diseases are a varied assortment of central nervous system disorders characterized by the gradual and progressive loss of neural tissue or nerve cells. A large number of such sporadic and hereditary neurodegenerative disorders are characterized neuropathologically by intracellular and/or extracellular aggregates of proteinaceous fibrils or amyloids (see Table 1.1). These lesions are not mere markers of the disease state but are directly implicated in progressive brain degeneration. Most often, aggregation is a result of abnormal protein-protein interactions (for review see Trojanowski et al., 1998). This is exemplified by the intranuclear neuronal inclusions that are formed by the aggregation of mutant proteins harboring abnormally expanded polyglutamine tracts in hereditary tri-nucleotide repeat disorders like Huntington's disease (HD), the intracytoplasmic neurofibrillary tangles (NFTs), as well as the extracellular amyloid or senile plaques (SPs) in sporadic and familial Alzheimer's disease (AD), and by the prion protein deposits in the brains of patients with a sporadic or genetic form of spongiform encephalopathy.

The sporadic AD and familial AD represent the heterogeneous ends of a disease spectrum that overlaps with a large group of disorders with prominent tau protein-rich tangle pathology in the brain known as "taupathies" on the one hand, and those with prominent α-synuclein-rich inclusion pathology in the brain known as "synucleinopathies" (see Table 1.1). In Taupathies, the protein aberrantly present in an

Table 1.1 Neurodegenerative diseases characterized by filamentous lesions formed from aggregated peptides/proteins

Disease	Lesion/Components	Location
Alzheimer's Disease (AD)	Senile Plaques/ β-Amyloid	Extracellular
	Neurofibrillary tangles/ PHFtau	Intracytoplasmic
Amyotrophic Lateral Sclerosis (ALS)	Spheroids/	Intracytoplasmic
Dementia with Lewy Bodies (DLB)	Lewy Bodies/ α-synuclein	Intracytoplasmic
Multiple System Atrophy (MSA)	Glial Cell Inclusions /α-synuclein	Intracytoplasmic
Neuronal intranuclear inclusion disease	Inclusions/Expanded polyglutamine tracts	Intranuclear
Parkinson's Disease (PD)	Lewy Bodies/ α-synuclein	Intracytoplasmic
Prion diseases	Amyloid plaques/Prion	Extracellular
Taupathies*	Neurofibrillary tangles/ AD-like PHFtau	Intracytoplasmic
Tri-nucleotide repeat diseases	Inclusions/Expanded polyglutamine tracts	Intranuclear
Lewy Body Variant Alzheimers disease (AD + DLB)	Senile Plaques/ β-Amyloid	Extracellular
	Neurofibrillary tangles/ PHFtau	Intracytoplasmic
	Lewy Bodies/NF α-synuclein	Intracytoplasmic

aggregated form is the microtubule-associated protein tau. Abnormal phosphorylation of tau results in a pre-tangle stage leading to the formation of filamentous polymers called paired-helical filaments (PHF). Aggregation of this PHF results in the formation of cytoplasmic (intracellular) NFT (for review see Avilla, 2000).

In Synucleinopathies, the protein α-synuclein is modified to form aggregates and is the major component of Lewy bodies. Although Lewy bodies (LBs) are regarded as hallmark intracytoplasmic neuronal inclusions of Parkinson's disease (PD), they also occur commonly in the brains of patients with the typical clinical and pathological features of AD (Hardy et al., 1998). Alzheimer's disease is also marked by the aggregation of dysfunctional β-amyloid –major component of senile plaques. This overlap is striking and underlines the notion of "fatal attractions" among brain proteins as a common mechanistic theme to account for brain degeneration in neurodegenerative disorders of the elderly (for review seeTrojanowski, et al., 1998). Strange enigma of these protein aggregation related disorders is that the familial form is caused by missense mutations in the gene encoding the disease protein forming the hallmark brain lesions while the sporadic form is caused by the corresponding wild type protein causing the same brain lesions.

Neurodegenerative disorders involving expanded polyglutamine tracts

Proteins containing expanded polyglutamine tracts in addition to already mentioned tau, α-synuclein and β-amyloid are responsible for two major groups of neurodegenerative disorders: neuronal intranuclear inclusion disease and the tri-nucleotide repeat disorders (see Table 1.1). A significant overlap exists between the two groups of disorders. In both cases, causative protein causes intraneuronal intranuclear inclusions. The latter group is more defined and discussed. The tri-nucleotide repeat

disorders can be dividied into polyglutamine (poly-Q) expansion disorders and non-polyglutamine expansion disorders. Huntington's disease is one of the diseases belonging to the polyglutamine expansion disorders involving CAG (Q) repeats while Friedreich Ataxia involving expansion of GAA (E) repeats and Fragile X syndrome involving expansion of CGG ® repeats are some examples of non-polyglutamine expansion disorders.

Poly-Q expansions in several unrelated proteins are responsible for atleast nine inherited neurodegenerative diseases (see Table 1.2). In all the cases, proteins are are expressed widely in brain and in other tissues, yet each is toxic in a different, highly specific group of neurons and produces a distinct pathology. All nine disorders show late onset of neurological symptoms with progressive neuronal dysfunction. As mentioned earlier, the characteristic feature of poly-Q diseases is the formation of insoluble, granular and fibrous deposits in affected neurons termed as neuronal inclusions (Perutz, et al., 1999). In 1994, Perutz proposed that long sequences of poly-Q might be able to

Table 1.2 Neurodegenerative disorders caused by poly-Q expansions

Disease Pathology	**Protein containing poly-Q expansion**	**Mode of inheritance**
Huntington's disease (HD)	Huntingtin	Autosomal
Spinobulbar muscular atrophy (Kennedy's disease)	Androgen receptor	X-linked
Dentatorubral pallidoluysian atrophy	Atrophin-1	Autosomal
Spinocerebellar ataxia (SCA) types 1,2,3,6,7 and 13	SCA 1,2,3,6,7 and 13 proteins	Autosomal

form stable β-hairpins (Perutz, et al., 1994). These structures also called “polar zippers” consist of poly-Q-containing β-strands held together by hydrogen bonds between both main-chain and side-chain amides. Poly-Q-containing hairpins may self-associate to form stable β-sheet aggregates with fibrillar morphology. Interestingly certain transcription factors containing poly-Q segments in the nonpathological range, such as TATA-binding protein (TBP) and CREB-binding protein (CBP) were detected in neuronal inclusions. Sequestration of these essential proteins via poly-Q-poly-Q interaction is suggested to cause neuronal toxicity (Perez et al., 1998; Kazantsev et al., 1999; Nucifora et al., 2001). Additional proteins detected in neuronal inclusions were ubiquitin (Ub), 19S and 20S proteasome complexes and several molecular chaperones (Paulson et al., 1997; Davies et al., 1997; Cummings et al., 1998; Chai et al., 1999; Waelter et al., 2001).

Huntington’s disease is one of the best known poly-Q disorders. Molecular genetics has revealed the disease trigger, an inherited unstable CAG expansion in the N-terminl exon1 of a novel 4p16.3 gene that lengthens a polyglutamine segment in the 350kDa protein huntingtin (Htt). The protein has unknown but essential function. The major characteristic of HD is a selective loss of neurons in the striatum and cortex leading to movement disorders, dementia, and eventually death. The toxicity of Htt in specific neurons correlates with the length of the glutamine expansion, but the mechanism of toxicity is unknown. When poly-Q tracts exceed the length of ~37 residues, disease ensues. The length of the poly-Q stretch inversely correlates with the time of onset of the disease and the time of formation of Htt aggregates (Martindale et al., 1998). Poly-Q expansion in Htt can cause cellular dysfunction either by sequestering important cellular proteins or could partially inhibit the Ubiquitin (Ub)-proteasome

pathway for protein degradation similar to what was proposed in general for poly-Q expansion disorders. These two mechanisms are not exclusive but may act in conjunction (Sakahira et al., 2002). N-terminal fragments of Htt with poly-Q extensions was indeed shown to be involved in numerous protein-protein interactions (for review see Li and Li., 2004). This fragment aggregated and was even more toxic than full length Htt when expressed in transgenic animals or cultured cells (Mangiarini et al., 1996; Davies et al., 1997). This suggests that at least some parameters of poly-Q associated aggregation and toxicity could be reproduced in the experimental assays using only N-terminal (exon 1) poly-Q expanded fragments of Htt.

Neurodegenerative disorders involving prion proteins

In 1982, Stanley Prusiner first coined the term 'Prion' to describe protein as a novel infectious agent. Prions are protein isoforms that reproduce themselves by converting normal protein of the same sequence into a prion state (Prusiner, 1982). Therefore, prion infection does not require transmission of any nucleic acid and is instead transmitted by the protein itself. The mammalian prion diseases like Bovine spongiform encephalopathy (BSE) also known as "Mad Cow Disease" affecting cattle, Scrapie affecting sheep, Gerstmann-Straussler-Scheinker Disease (GSS) and Creutzfeldt-Jacob Disease (CJD) affecting human are characterized by the accumulation of the PrP protein. The normal protein designated as PrP^{C} and the pathological or infectious form designated as PrP^{SC} have the same genetic structure but differ in their structure as mentioned previously. Circular dichroism and NMR analysis indicated that PrP^{C} consists of mostly α-helical structures, while PrP^{SC} has high β-sheet content (Heller, 1996; Nguyen et al., 1995). PrP^{SC} molecules form large fibrillar amyloid structures called amyloid plaques and this ability is attributed to the high β-sheet content.

Thus etiology of other human degenerative diseases like Alzheimer's, Huntington's etc shares a remarkable similarity with the prion diseases ie ability of the causative protein to form amyloid plaques.

Yeast Prions

Prion Criteria

The three extensively studied non-mendelian prion elements in *S cerevisiae* are [PSI^+], [URE3] and [RNQ^+]. Yeast prions are passed from one yeast cell to another through the cytoplasm, either from mother to daughter cell during budding, from parent diploid to spore clones during meiosis or through cytoplasmic mixing during mating. In addition to these three prions, several other 'candidate prions' are identified using genetic and biochemical methods, eg [NU^+] (Sondheimer and Lindquist, 2000; Santoso et al., 2000; Derkatch et al., 2001). Table 1.3 gives details of the features of these yeast prions.

A non-Mendelian element can be considered prion only if it satisfies several

Table 1.3 Yeast prions and their features

Gene name	Wild-type protein	Normal cellular function	Prion designation	Prion forming domain (PFD)	%Gln + Asn in PFD
SUP35	Sup35	Translation termination	[PSI^+]	aa 1-123	43
RNQ1	Rnq1	Unknown	[RNQ^+]	aa 153-405	43
URE2	Ure2	Nitrogen catabolism	[URE3]	aa 1-89	47
NEW1	New1	Unknown	[NU^+]	aa 1-153	26

criteria (Wickner, 1994). The spontaneous appearance of prions in yeast occurs at a very low rate, on the order of 10^{-6} in a given population. The first criteria that must be met is that overexpression of the normal cellular counterpart of a prion protein must increase the rate of prion induction. Second, once the prion forms, it should be self-propagating and transmissible. In yeast, this means it should be transmitted through mitosis, meiosis, and during mating. In addition, there is a relationship between the chromosomal copy of the gene coding for the prion protein and the prion phenotype, that is, prion proteins are not completely independent of the gene that codes for them. Mutations in the prion gene leads to alterations of the prion phenotype, and elimination of that gene or its prion forming domain will eliminate the prion as well. Finally, all yeast prions described to date are reversibly cured by millimolar quantities of non-mutagenic agents such as guanidine hydrochloride (GuHCl).

Yeast prion [PSI$^+$]

The yeast prion [PSI$^+$] is an aggregated form of Sup35 that was first identified as a factor which increased efficiency of readthrough of stop codons in a process called nonsense suppression (Cox, 1965). Sup35 functions with Sup45 to identify stop codons and terminate translation. Sup35 protein is essential such that cells lacking a functional copy of *SUP35* are not viable. The reporter system used to assay the presence of [PSI$^+$] in the cell relies on its basic function of translation termination. [PSI$^+$] causes nonsense suppression of a premature stop codon present in the allele of *ADE1* gene (*ade1-14*$_{UGA}$). [psi$^-$] cells referring to PrP^C state contain soluble functional form of Sup35 that can terminate translation. In contrast to a [psi$^-$] cell, Sup35 produced in a [PSI$^+$] cell is sequestered into an aggregated form, increasing the level of nonsense suppression of the *ade1-14* $_{UGA}$ mutation enabling them to generate Ade1 product (Figure 1.1 A). Thus [PSI$^+$] cells are able to grow on medium lacking adenine (-Ade medium). The [PSI$^+$] cells

appear white in color when grown on rich medium like YPD due to the accumulation of the white pigment, on the other hand, the [psi$^-$] cells appear red on YPD and cannot grow on –Ade medium (Figure 1.1 B). In [PSI$^+$] cells, majority of Sup35 is found in the aggregated (insoluble) fraction, while majority of Sup35 in [psi$^-$] cells is detected in the soluble fraction (Patino et al., 1996; Paushkin et al., 1996) as depicted in Figure 1.1 C.

The Sup35 protein of 685 aa could be subdivided into three regions: N-proximal or Sup35N, middle or Sup35M, and C-proximal or Sup35C. The Sup35N region of 123 aa, rich in glutamines (Q) and asparagines (N), which is common to another yeast prion [URE3] and to the Huntington disease protein huntingtin is dispensable for termination function but solely responsible for the formation and propagation of [PSI$^+$]. It also contains tandem imperfect oligopeptide repeats (PQGGYQQYN) similar to those (PHGGGWGQ) of the mammalian prion determinant PrP (Kushnirov et al., 1988). In both cases, the repeat expansion is known to increase the frequency of prion conversion (Prusiner and Scott, 1997; Liu and Lindquist, 1999). All prions known to date are protein aggregates, believed to transmit the aggregated state via the "seeded" or "nucleated" polymerization process (Lansbury and Caughey, 1995). In vitro (and at least in certain cases in vivo), prion aggregates were shown to possess properties of amyloids, highly ordered fiber-like structures (Prusiner, 1997; Chernoff, 2001; Wickner et al., 2001).

Yeast prion [PIN$^+$]/[RNQ$^+$]

The presence of [PIN$^+$] is required for [PSI$^+$] inducibility ie it is necessary for [PSI$^+$] to appear spontaneously or following induction by excess Sup35N (Derkatch et al., 1997). [PIN$^+$] is required at the step of [PSI$^+$] initial occurence but is not needed for its propagation. [PIN$^+$] appears to be a prion as it passes the prion criteria that are mentioned above 1) Like the established yeast prions [PSI$^+$] and [URE3], it is inherited in

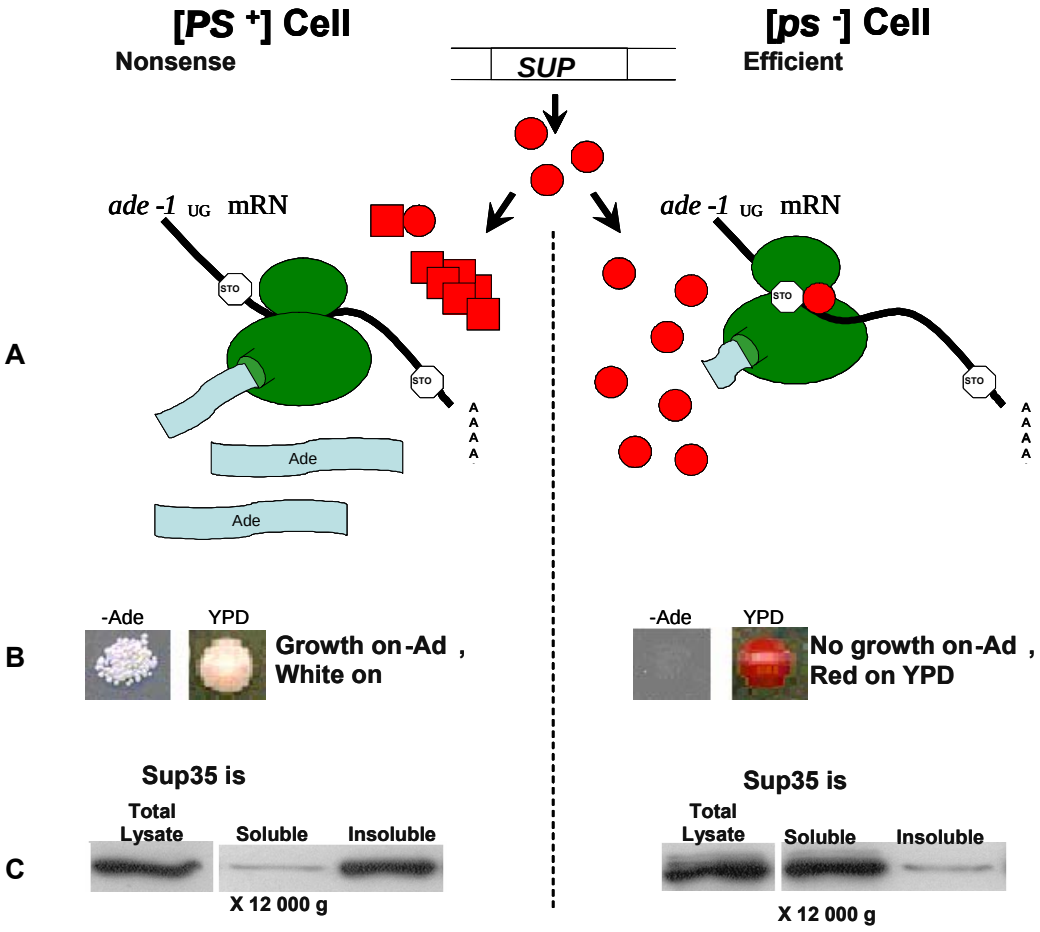


Figure 1.1 Experimental assay used to monitor presence of the yeast prion [PSI$^+$] A. Sup35 protein is produced at the same level in both [psi$^-$] and [PSI$^+$] cells. The *ade1-14*$_{UGA}$ reporter system, which contains a premature stop codon, is commonly used to assay presence of [PSI$^+$]. In a [psi$^-$] cell, Sup35 remains soluble and therefore readily available to participate in activities related to translation termination. In a [PSI$^+$] cell, Sup35 is sequestered into an aggregated form, increasing readthrough of nonsense codons by translating ribosomes. In contrast to [psi$^-$] cells, [PSI$^+$] cells are able to generate significant quantities of the Ade1 product from the *ade1-14*$_{UGA}$ transcript. B. Accumulation of Ade1 is manifested in [PSI$^+$] cells as growth on –Ade medium and a white color on YPD. Absence of the Ade1 product is indicated in [psi$^-$] cells as absence of growth on –Ade medium and red color on YPD. C. The majority of Sup35 can be found in the aggregated (insoluble) fraction of [PSI$^+$] cell lysates, and the soluble fraction of [psi$^-$] cell lysates prepared by differential centrifugation.

a non-mendelian manner 2) It depends on Hsp104 (chaperone discussed later) for propagation 3) It is cured by growth in the presence of low levels of guanidine hydrochloride (GuHCl) and 4) reappears in cured strains (Derkatch et al., 2000). Also like [PSI^+] (Derkatch et al., 2000) and [URE3] (Fernandez-Bellot et al., 2000) newly appearing [PIN^+] elements are frequently unstable. Presence of Rnq1 or Ure2 prion aggregates made yeast strains [PIN^+] and the spontaneous appearance of [PIN^+] was accompanied by the appearance of Rnq1 aggregates (Derkatch et al., 2001) suggesting that the [PIN^+] factor corresponds to the prion form of Rnq1. The function of Rnq1 is not known. Unlike all other prions where prion forming domain (PFD) is mostly located in the N-terminal region of the protein, the PFD of Rnq1 is located towards the C-proximal region of the protein spanning aa 153 to 405 (Sondheimer and Lindquist, 2000).
Two models were proposed to explain the dependence of [PSI^+] on [PIN^+]. In the first model, pin acts as an inhibitor, in its non-prion conformation it inhibits *de novo* formation of [PSI^+]. The second model predicts that [PIN^+] aggregates provide an initial nuclei facilitating [PSI^+] appearance. Just as is seen with [PSI^+], the mutant huntingtin is toxic and can aggregate only in the presence of [PIN^+] (Meriin et al., 2002) supporting second model. But examples do exist where multiple prions result in antagonistic relations (Bradley et al., 2002; Schwimmer and Masison, 2002).

Similarities between yeast prion proteins and huntingtin Htt

The ability to form large fibrillar amyloid like structures is a feature shared by the poly-Q containing proteins such as huntingtin and the yeast prions. Concurrently, the amino-terminal huntingtin fragments form SDS-resistant aggregates similar to the yeast prion protein Sup35 fibrils.

Yeast prion proteins and candidate prion proteins are characterized by unusually high Gln and Asn content. The average Gln + Asn content for a yeast protein is 9%

(Santoso et al., 2000). The prion forming domains of yeast prions Sup35, Ure2, Rnq1 and New1 possess Gln + Asn of 26 to 47%. The repetitive sequences of Gln (Q) and Asn (N) have been shown to form β-sheets and 'polar zippers' (DePace et al., 1998; Perutz et al., 1994) as seen with the poly-Q domain of Htt. Structural similarities between the three proteins- Htt, Sup35 and Rnq1 are shown in figure 1.2.

Sup35 N-terminal (N) domain is required for [PSI^+] propagation (Ter-Avanesyan et al., 1993; Ter-Avanesyan et al., 1994). Overexpression of Sup35 or Sup35N induces de novo formation of [PSI^+] (Chernoff et al., 1992; Chernoff et al., 1993; Ter-Avanesyan et al., 1993; Derkatch et al., 1996). This most likely occurs due to the fact that the excess Sup35 increases the probability of the protein to adopt the self-perpetuating prion isoform in the presence of already existing prion. The prion aggregates can self-seed the conversion of normal Sup35 into the prion form and do not require continued expression of Sup35 to maintain [PSI^+] (Chernoff et al., 1993). Continued expression of Sup35 in a [PSI^+] cell is toxic (Chernoff et al., 1992; Ter-Avanesyan et al., 1993). Similarly, excess mutant huntingtin also causes toxicity in yeast cell (Meriin et al., 2002). The similarities between the prion proteins and huntingtin suggest common players in their life-cycle.

Dependence of HttPoly-Q toxicity on endogenous yeast prions in the yeast model

Yeast model has provided significant insight into the understanding of neurodegenerative disorders like prion disease, Parkinson's disease, polyglutamine expansion disorders,etc. Genetic experiments in mice, which are indispensable for studying the molecular basis of neurological disorders, have certain limitations that include slow pace and high costs. It is therefore not surprising that in recent years numerous neurological diseases have been modeled in genetically tractable organisms, including Drosophila, Caenorhabditis elegans, and yeast. Yeast models in particular

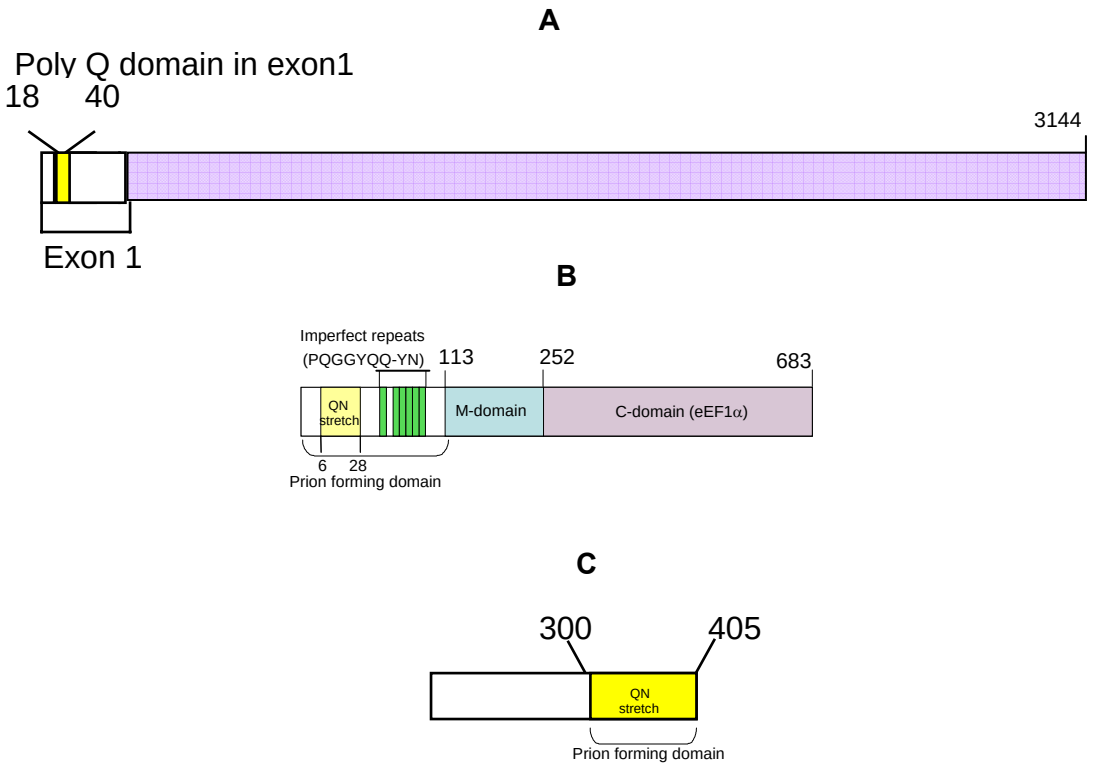


Figure 1.2 Structural organization of Q-rich proteins
A. Structural organization of huntingtin protein (Htt). Htt is a 350 kDa protein comprising 3144 amino acids. The exon 1 of Htt contains a Q-rich domain from amino acids 18 to 40. B. Structural organization of Sup35 Sup35 protein is a 77 kDa protein comprising of 683 amino acids. It contains three structural domains. The N domain (prion forming domain) is 53% Gln (Q) -rich. It is also rich in Asn (N), Gly (G) and Pro (P) residues and contains multiple imperfect repeats. The middle domain (M) is highly charged and the C terminal domain is the functional domain responsible for translation termination. C. Structural organization of Rnq1. Rnq1 protein is 42.5 kDa comprising of 405 amino acids. The C terminal end (prion forming domain) of this protein is 46% Gln-rich.

have a special advantage with respect to genome-wide experimental approaches as a result of the completed sequencing of the genome, the availability of a collection of precise deletion mutants of every gene in the genome, and the rapidly evolving databases of yeast protein-protein interactions and gene expression patterns. These large and easily accessible bodies of information, coupled with the ease with which yeast can be manipulated genetically, have led to dissection of novel mechanisms of neurodegenerative disorders.

HttPoly-Q aggregation and toxicity have been studied by using simple organisms such as fruit flies (Jackson et al., 1998), *Caenorhabditis elegans* (Faber et al., 1999) and yeast *Saccharomyces cerevisiae*. Yeast assays usually employ short constructs derived from the poly-Q expanded exon 1 of Htt. The GFP-tagged polypeptides from normal (Q25) and mutant (Q103) forms of huntingtin are expressed under galactose-inducible promoter (figure 1.3 A). While poly-Q aggregation has been readily observed in yeast (Krobitsch and Lindquist,2000; Muchowski et al., 2000; Cao et al, 2001; Kimura et al., 2001; Muchowski et al., 2002), a yeast-based assay for poly-Q toxicity has not been available until recently. It turned out that efficient cytoplasmic aggregation and toxicity of the chimeric protein, containing the poly-Q expanded exon 1 of Htt fused to the green fluorescent protein (GFP), could be detected only in the yeast strains bearing an endogenous yeast QN-rich protein, Rnq1, in its prion form, called [RNQ^{+}], or [PIN^{+}] (Meriin et al., 2002) shown in figure 1.3 B, C. In the absence of a prion, poly-Q aggregates are rarely found, and toxicity is not seen. Yeast prions apparently “seeds” aggregation of the heterologous poly-Q protein (Figure 1.3 D). Prion form of QN-rich domain of the other yeast protein, New1, has also been shown to facilitate aggregation of poly-Q construct originated from the other mammalian protein, mutant ataxin-3 involved in Machado-Joseph Disease (MJD), although cell toxicity of that protein was not detected in yeast (Osherovich and Weissman, 2001). Further analysis has demonstrated

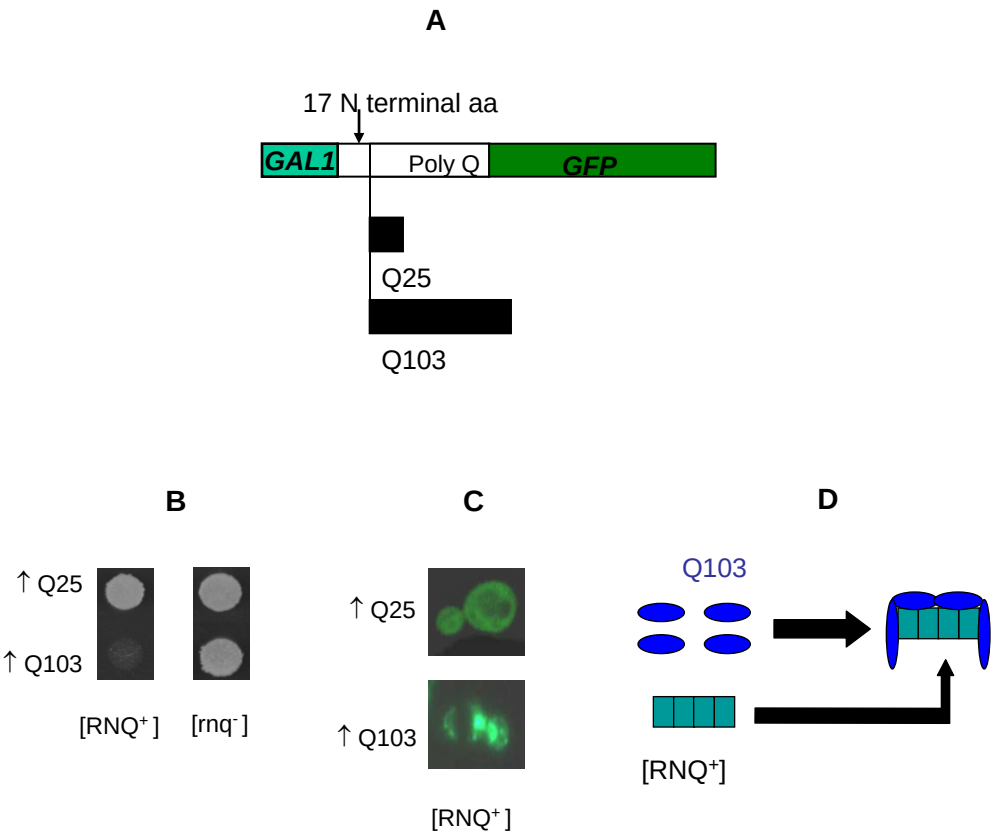


Figure 1.3 Poly-Q associated phenotypes in prion containing yeast
A. Cartoon of the huntingtin construct expressed in yeast. Polypeptides from normal (Q25) and mutant (Q103) forms of huntingtin fused to GFP are expressed under galactose-inducible promoter B. Q103 cytotoxicity depends on the presence [RNQ$^+$] prion. Yeast expressing Q103 do not show growth on galactose selective medium in the presence of [RNQ$^+$] prion but show growth in [rnq$^-$] yeast. Q25 expressing yeast can grow on galactose containing selective medium irrespective of the presence or absence of the [RNQ$^+$] prion. C. Excess Q103GFP forms clumps while excess Q25GFP shows diffused fluorescence. Yeast expressing Q103 forms aggregates only in the presence of [RNQ$^+$] while yeast expressing Q25 show diffused fluorescence irrespective of the presence or absence of the [RNQ$^+$] prion. D. Model proposing the role of pre-existing prions in inducing aggregation of mammalian polyglutamine proteins in yeast. Prions provide initial nuclei for other Q-rich aggregates.

that prion-dependent toxicity of the Htt-derived poly-Q construct in yeast is associated with the defect of endocytosis, possibly via sequestration of some components of the vesicle-assembly machinery by poly-Q aggregates (Meriin et al., 2003). As subcellular localization of Htt in mammalian cells has led to a suggestion that it may play a role in vesicle trafficking (Muchowski et al., 2002), it is possible that yeast model reflects certain features of cell toxicity that are relevant to mammalian HD.

Proteins and processes modulating the biological effects of Q-rich aggregates of Sup35 and Htt

Molecular chaperones

Molecular chaperones are proteins which can recognize and bind to non-native (denatured, misfolded intermediates) polypeptides to facilitate them to fold to their native states that are specified by their primary sequences. In cytosol where protein concentrations are high, chaperones are required for protein folding, especially for large multidomain protein folding at elevated temperature. Two types of molecular chaperones function to facilitate protein folding- 1) Heat shock proteins (Hsp)- These are proteins that suppress polypeptide aggregation and promote protein folding and 2) Enzymes like DsbA, DsbB, PDI that catalyse or correct the disulphide bond formation.

The major families of Hsps modulating the Q-rich aggregates are Hsp100, Hsp70 and Hsp40. Hsp104, a member of the ClpB/Hsp100 family facilitates the ATP-dependent resolubilization of misfolded and aggregated proteins in conjunction with Hsp70/DnaK and Hsp40/DnaJ (Glover and Lindquist,1998). In yeast, Arabidopsis and maize, the Hsp100/ClpB play a major role in acquisition of thermotolerance ie the ability to survive very high temperatures (Sanchez and Lindquist, 1990; Queitsch et al., 2000; Nieto-Sotelo et al., 2002). For eg: Yeast cells expressing Hsp104 (yeast Hsp100) are 1000-times more viable after exposure to temperatures $\geq 50°C$ or to 20% ethanol than cells

carrying deletions of *HSP104* (Sanchez and Lindquist, 1990; Sanchez et al., 1992). This capacity is attributed to Hsp104's ability to resolubilize and refold aggregated proteins damaged by protein-damaging stress that includes heat stress as already mentioned (Glover and Lindquist, 1998; Parsell et al., 1994; Goloubinoff et al., 1999). During times of severe stress, rate of protein aggregation exceeds the capacity of other heat shock proteins to prevent aggregate accumulation and Hsp104 becomes critical for survival. This explains why Hsp104 is not required for normal growth or even growth at higher temperatures but is crucial for cell survival at extreme temperatures. Hsp104 levels increase in late exponential and stationary phase of growth, as well as during sporulation (Sanchez and Lindquist, 2000; Sanchez et al., 1992).

Hsp70 (DnaK) family functions in an ATP-dependent stabilization of hydrophobic regions of extended polypeptides. Yeast Hsp70 has two major subfamilies Ssa and Ssb. The Ssa subfamily is one of the major heat shock inducible cytosolic chaperone subfamilies in yeast. This subfamily has four members Ssa1, 2, 3 and 4. Presence of atleast one Ssa is required for vegetative growth (Werner-Washburne et al., 1989). while Ssb proteins are associated with translating ribosomes. The Ssb subfamily is composed of two nearly identical proteins Ssb1 and Ssb2, which are not essential for viability and not inducible by heat shock (Nelson et al., 1992).

Hsp40 is a regulator of Hsp70 (Cyr and Douglas, 1994). The J-domain of Hsp40 can stimulate the ATPase activity of Hsp70 in order to refold non-native polypeptides. Hsp40 can bind non-native polypeptides directly and deliver them to Hsp70 for folding (Qian et al., 2002 for example).

Role of molecular chaperones in prion propagation

Yeast prion propagation is modulated by the Hsp104/Hsp70/Hsp40 complex. Prion propagation operates at the level of protein folding and thus chaperone proteins

that assist protein folding come into the picture. Both overexpression of Hsp104 and deletion of Hsp104 cures [PSI$^+$] (Chernoff et al., 1995). Infact intermediate levels of Hsp104 are required to maintain [PSI$^+$]. In accordance, overproduction of Hsp104 in [PSI$^+$] cells results in shift of Sup35 from the insoluble (aggregated) to soluble (non-aggregated) fraction (Patino et al., 1996; Paushkin et al., 1996). Hsp104 is also involved in the propagation of other yeast prions, interestingly overexpression of Hsp104 does not cure yeast prions [URE3] (Moriyama et al., 2000) or [RNQ$^+$] (Derkatch et al., 1997; Sondheimer and Lindquist, 2000).

Hsp70-Ssa1 protects [PSI$^+$] from curing effects of excess Hsp104 (Newnam et al., 1999) and facilitates de novo induction of the [PSI$^+$] prion in [psi$^-$] cells (Allen et al., 2004). Atleast in some [PSI$^+$] variants, excess Ssa1 also increases phenotypic expression of [PSI$^+$], detected as nonsense-suppression (Newnam et al., 1999). Ssa1 was also shown to be important for the mitotic stability of [PSI$^+$] (Jung et al., 2000; Jones and Masison, 2003). Although Ssa1 does not cure endogenous [PSI$^+$], some chimeric constructs of [PSI$^+$] were shown to be cured by Hsp70-Ssa1 and Hsp70-Ssb1 (Kushnirov et al., 2000). In contrast to Ssa1, Ssbs antagonize [PSI$^+$]. Excess Ssb increases [PSI$^+$] curing by overexpressed Hsp104 (Chernoff et al., 1999). In the *ssb1Δssb2Δ* strain, spontaneous [PSI$^+$] formation in [psi$^-$ PIN$^+$] cells is increased and curing of [PSI$^+$] by excess Hsp104 is decreased (Chernoff et al., 1999). Some [PSI$^+$] isolates can also be cured by excess Ssb (Chernoff et al., 1999; Kushnirov et al., 2000).

Hsp40-Sis1 regulates the maintenance of [RNQ$^+$] (Sondheimer et al., 2001) and excess Hsp40-Ydj1 results in gradual loss of [URE3] (Moriyama et al., 2000). Some data also suggest a role for Hsp40 in [PSI$^+$] propagation (Jones and Masison, 2003; Kushnirov et al., 2000).

Role of molecular chaperones in poly-Q aggregation and toxicity

Molecular chaperones represent one group of proteins frequently recruited to poly-Q inclusions. Their presence in aggregates suggests that expanded poly-Q tracts are recognized as misfolded conformers and that cellular quality-control mechanisms are activated in an attempt to prevent their accumulation (Sherman and Goldberg, 2001). As at least some models confirm relationship between poly-Q aggregation and cell toxicity, chaperone proteins counteracting aggregation are well positioned as likely antagonists of the poly-Q disorders. Indeed, some chaperones of the evolutionary conserved Hsp70 and Hsp40 families counteracted poly-Q aggregation *in vitro* (Muchowski et al., 2000) and *in vivo*, as seen in the cultured mammalian cells, and in the fruit fly assays (Kazemi-Esfarjani and Benzer, 1999; Warrick et al., 1999; Chan et al., 2000; Cummings et al, 2001; Sakahira et al., 2002).

In yeast, aggregates of the heat damaged proteins are solubilized and refolded by the complex of chaperone proteins, including Hsp104, Hsp70 and Hsp40 (Glover and Lindquist, 1998). Overproduction of some members of the Hsp70 and Hsp40 families was shown to counteract poly-Q aggregation in the yeast cells (Muchowski et al., 2000). Simultaneous deletion of the *SSA1* and *SSA2* genes, coding for the yeast proteins of the Hsp70 family, as well as a point mutation in Ydj1, a protein of the Hsp40 family, also decreased poly-Q aggregation in yeast (Meriin et al., 2002). Hsp104 was shown to be essential for the aggregation of poly-Q in yeast, and its deletion or overproduction eliminated or reduced poly-Q aggregation at least in some assays (Krobitsch and Lindquist, 2000; Satyal, et al., 2000; Cao, et al., 2001; Kimura, et al., 2001).

Ubiquitin-Proteasome system

Along with molecular chaperones, Ubiquitin/Proteasome pathway is responsible for dealing with misfolded proteins usually by proteolysis. Proteolysis can occur in yeast

vacuole or in the cytosol. Protein destruction in cytosol is achieved through the action of proteasome complex. Proteins marked for destruction are tagged with Ubiquitin. Ub-mediated proteolysis could play a role in prion propagation by regulating the free substrate available to be converted into prion aggregates. Importance of Ubiquitin conjugating enzyme (Ubc4) in [PSI$^+$] propagation has been shown by K. Allen (former member of Chernoff Lab).

The finding that poly-Q inclusions stain positively for ubiquitin, 19S and 20S complexes suggested that the Ub-proteasome pathway may be involved in poly-Q pathogenesis (Cummings et al., 1998; Chai et al., 1999). There are several reports that formation of poly-Q inclusions is accelerated when proteasome –inhibitors are added to transfected cells (Cummings et al., 1998; Chai et al., 1999;). Intracellular aggregation of poly-Q proteins has been proposed to impair the ubiquitin-proteasome system (for review see Sherman and Goldberg, 2001).

Cytoskeleton-associated proteins

Cytoskeletal assembly proteins have been shown to modulate prion propagation as well as play a role in poly-Q disorder like Huntington's disease. Sla1 and Sla2 are two important members of the cytoskeletal family. Sla1 along with Las17 are important in actin patch assembly while Sla2 plays an important role in the polarization of the cortical actin cytoskeleton. Sla2 is a homolog of mammalian Hip1 (huntingtin-interacting protein 1) (Holtzman et al., 1993) which is known to function in endocytic processes. Sla1 and Sla2 both have some domain structures reminiscent of prion-forming domains. Sla1 contains oligopeptide repeats (Holtzman et al., 1993) and Sla2 has a region rich in Gln and Asn residues (Michelitsch and Weissman, 2000), but neither can form prions themselves. The protein Sla1 was shown to interact with Sup35N in yeast two-hybrid screen and [PSI$^+$] induction by excess Sup35 was reduced in the *sla1Δ* background

(Bailleul et al., 1999). The cytoskeletal inhibitor latrunculin-A (LatA) was shown to be able to cure [PSI^+], but not [PIN^+] (Bailleul-Winslett et al., 2000).

Several genes required for early endocytic events and organization of cortical actin patches when mutated or deleted, enhanced Q103 toxicity establishing a link between poly-Q toxicity and inhibition of early steps of endocytosis (Meriin, et al., 2003). Eg: Sla1, Pan1, Sla2. Growth of cells expressing Q103 was impaired to a greater extent in early endocytic mutant cell background than that of wild-type cells. Sla1 was also shown to interact with extended poly-Q in two-hybrid screen (Bailleul et al., 1999). This enhanced poly-Q toxicity required [RNQ^+] dependent aggregation of poly-Q (Meriin, et al., 2003).

Objectives

The goal of this study was to find factors that were important in the life-cycle of Q-rich protein aggregates. This was achieved by studying the interaction between chaperone system, ubiquitin system, cytoskeletal system and Q-rich proteins causing some aggregation-related disorders using the yeast prion experimental model. This study would thus be helpful in providing valuable information towards development of therapy for some aggregation-related disorders and in possibly understanding the mechanisms underlying them.

CHAPTER 2

GENERAL MATERIALS AND METHODS USED FOR YEAST MOLECULAR BIOLOGY

Materials

Yeast strains

All yeast strains used in this study are listed in Table 2.1. More detailed descriptions of individual strains are available for some strains in the corresponding chapter in which the strain was used. The presence of the [PIN^+] – prion form of the Rnq1 protein is designated [RNQ^+] or [PIN^+] interchangeably.

The most commonly used strains in this work are a set of 4 isogenic derivatives of yeast strain 74-D694, which differ only in their prion composition. Strain 74-D694 ([psi^- PIN^+]) is referred to as OT60. The weak [PSI^+ PIN^+] strain is OT55 and the strong [PSI^+ PIN^+] strain is OT56. They were independently induced by overexpression of *SUP35* in strain OT60 (Derkatch, et al., 1996). Strong [PSI^+] strain is more efficient in showing nonsense suppression of the *ade1-14*$_{UGA}$ mutation resulting in growth on –Ade medium after 3-4 days and a white/light pink color on YPD, compared to the 7-8 day incubation time required to see growth of weak [PSI^+] cells on –Ade, and a pink color on YPD. GT17 is a [psi^- pin^-] derivative acquired by GuHCl treatment of strain OT56 as described previously (Derkatch, et al., 1997).

The other most commonly used set of isogenic strains in these studies are derived from the strong [PSI^+ PIN^+] diploid parent GT81 (Chernoff, et al., 2000). GT81 is a self-diploid GT81 is heterozygous by *MAT* locus and homozygous by all other genes. GT81-1C and GT81-1D are haploid meiotic segregants of opposite mating types derived from GT81 (Chernoff, et al., 1999). GT234 (Chernoff, et al., 1999) and GT409 (Kim Allen

Table 2.1 Alphabetical list of *Saccharomyces cerevisiae* strains

Strain (Synonyms)	Genotype
GT17	*MAT***a** *ade1-14*$_{UGA}$ *his3-Δ200 leu2-3,112 trp1-289*$_{UAG}$ *ura3-52* [psi$^-$ pin$^-$]
GT81	*MAT***a***/ MAT*α *ade1-14/ade1-14 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 lys2/lys2* [PSI$^+$ PIN$^+$]
GT81-1C	Strong [PSI$^+$ RNQ$^+$] *MAT***a** haploid meiotic spore clone of GT81
GT81-1D	Strong [PSI$^+$ RNQ$^+$] *MAT*α haploid meiotic spore clone of GT81
GT146	*MAT* **a** *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ssb1::HIS3 ssb2::URA3* [PSI$^+$ RNQ$^+$]
GT157	[psi$^-$ PIN$^+$] derivative of GT146
GT159	*MAT***a** [psi$^-$ RNQ$^+$] haploid derivative of GT81
GT202 and GT203	[PSI$^+$] derivatives of GT157 Induction by CEN-GAL-SUP35
GT215-8A	*MAT* α *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 Δsup35::HIS3* [SUP35Sc URA3 CEN] [PSI$^+$]
GT234	*MAT*α *ade1-14*$_{UGA}$ *his3-Δ200 leu2-3,112 trp1-289*$_{UAG}$ *ura3-52 lys2* [psi$^-$ rnq$^-$]
GT255-2A	*MAT*α *ade1-14*$_{UGA}$ *his3-Δ200 lys2 ura3-52 leu2-3,112 trp1-289*$_{UAG}$ *sup35Δ::HIS3* [pASB2-CEN LEU2 SUP35] [psi$^-$ PIN$^+$]
GT310	*MAT* **α** *ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52 Δsup35::HIS3* [SUP35NMPm-CSc *URA3*] [PSI$^+$]
GT349	*MAT***a** *ubc4Δ::HIS3* derivative of GT81-1C
GT385	Heterozygous *ubc4Δ::HIS3 ubc5Δ::HIS3* derivative of GT340 and GT349 (isogenic to GT81) [PSI$^+$ PIN$^+$]
GT385-13A	*MAT*α *ubc4Δ* [psi$^-$ rnq$^-$] haploid meiotic spore clone of GT385
GT386	*MAT***a** *ubc4Δ::HIS3* [psi$^-$ PIN$^+$] derivative of GT349
GT387	*MAT***a** *ubc4Δ::HIS3* [psi$^-$ pin$^-$] derivative of GT349
GT393	*MAT*α *ade1-14 his3 lys2 ura3 leu2 trp1 sup35::HIS3* [PSI$^+$] [pASB2] [PSI$^+$ PIN$^+$]
GT490	[PSI$^+$ pin$^-$] derivative of OT56
GT532	*ubc4Δ* / ubc4*Δ* diploid (GT385-13A X GT580)
GT532-9B,-9D	*MAT***a** and *MAT*α haploid meiotic spore clones of GT532
GT534	*ubc4Δ* / ubc4*Δ* diploid(GT385-13A X GT582)
GT534-2A,2B	*MAT***a** and *MAT*α haploid meiotic spore clones of GT534
GT557	GuHCl cured strain of GT310 [psi$^-$ pin$^-$]
GT564	*MAT* **a** *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 rnq Δ: HIS3* [psi$^-$ PIN$^+$] rnq disruptant of GT159
GT573	*AQT-2 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] anti-polyQ toxicity derivative of GT349
GT574	*AQT-7 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] anti-polyQ toxicity derivative of GT349
GT575	*AQT-9 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] anti-polyQ toxicity derivative of GT349
GT579	*AQT-2 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pRS316GAL]
GT580	*AQT-2 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pYES2-103Q]

Table 2.1 (continued)

GT581	*AQT-7 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pRS316GAL]
GT582	*AQT-7 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pYES2-103Q]
GT592	[psi$^-$ PIN$^+$] derivative of GT310 obtained by WT Hsp104 treatment
GT606	*ade1-14/ade1-14 Δubc4::HIS3/Δubc4::HIS3 his3-Δ200(or -11, 15)/ his3-Δ200(or -11, 15) leu2-3,112/leu2-3,112 lys2/lys2 trp1-Δ/trp1-Δ ura3-52/ura3-52* [PSI$^+$]
GT607	*AQT-9 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pRS316GAL]
GT608	*AQT-9 MAT***a** *ubc4Δ::HIS3* [PSI$^+$ PIN$^+$] [pYES2-103Q]
GT675	*AQT-2 MAT***a** *ubc4Δ::HIS3* [psi$^-$ PIN$^+$]
GT676	*AQT-7 MAT***a** *ubc4Δ::HIS3* [psi$^-$ PIN$^+$]
GT677	*AQT-9 MAT***a** *ubc4Δ::HIS3* [psi$^-$ PIN$^+$]
GT724/GT756	*AQT-9 ubc4Δ* / ubc4*Δ* diploid [PSI$^+$ PIN$^+$] [pYES2-103Q]
GT724-3B,-3D	*MAT***a** and *MAT*α haploid meiotic spore clones of GT724
GT726	GT532-9B and GT532-9D [pYES2-103Q, YEP13]
GT727	GT534-2A and GT534-2B [pYES2-103Q, YEP13]
GT728	GT724-3B and GT724-3D [pYES2-103Q, YEP13]
GT729	*AQT-2/AQT-7 ubc4Δ* / ubc4*Δ* diploid [PSI$^+$ PIN$^+$]
GT730	*AQT-7/AQT-9 ubc4Δ* / ubc4*Δ* diploid [PSI$^+$ PIN$^+$]
GT741	*AQT-2/AQT-9 ubc4Δ* / ubc4*Δ* diploid [PSI$^+$ PIN$^+$]
GT769	*MAT***a** *ubc4Δ::HIS3* [psi$^-$ pin$^-$] derivative of *AQT-2* GT573
GT770	*MAT***a** *ubc4Δ::HIS3* [psi$^-$ pin$^-$] derivative of *AQT-7* GT574
GT771	*MAT***a** *ubc4Δ::HIS3* [psi$^-$ pin$^-$] derivative of *AQT-9* GT575
GT784-8C	*MAT* α *ade1-14 his3-Δ200 or 11,15 leu2-3,112 lys2 trp1-Δ ura3-52 ubc4::HIS3 rnq1::HIS3* [PSI$^+$]
GT818	*MAT***α** *ubc4Δ::HIS3, rnq1Δ::HIS3, leu2-3,112,lys2,trp1- Δ,ura3-52,ade1-14*
OT37	*MAT* α *his4 lys2*
OT38	*MAT* **a** *his4 lys2*
OT55 ([PSI$^+$]1-1-74-D694)	Weak [PSI$^+$ PIN$^+$] derivative of OT60
OT56 ([PSI$^+$] 7-74-D694)	Strong [PSI$^+$ PIN$^+$] derivative of OT60
OT59 alias PJ69-4A	*MAT* **a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ Gal2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-LaZ*
OT60 ([psi$^-$]-74-D694)	*MAT***a** *ade1-14$_{UGA}$ his3-Δ200 leu2-3,112 trp1-289$_{UAG}$ ura3-52* [psi$^-$ PIN$^+$]

from Y.O. Chernoff's lab) are meiotic segregants of GT81, cured of [PSI^+] and [PIN^+] by GuHCl. GT159 is the [psi^- PIN^+] derivative of a GT81 meiotic segregant, cured of [PSI^+], but not [PIN^+], by excess Hsp104 (Chernoff, et al., 1999).

Plasmids

Table 2.2 describes most of the plasmids used in this work. Listed separately (Table 2.3) are the plasmids with chimeric genes and in (Table 2.4) are the basic cloning vectors frequently employed as "empty vector" controls. Plasmid types are described as either *CEN* (centromeric) or 2μ (high copy number). Some experiments utilized entire sets of specialized constructs. For example the *SSA-SSB* chimeric constructs, *SSB* mutants, Hsp104 mutants given by S. Lindquist and yeast 2-hybrid constructs. They do not appear in tables but are explained in text in this section. Plasmids that were constructed for this study are mentioned in the appropriate chapter.

The *HIS3CEN* based plasmids used in chapter 3 : pH28-A509Db (Hsp104-A509D), pH28-2nd NBFb, pH28-monitorb, pH28-AAb, pH28-GTb, pH28-ISNBDDDb(Hsp104-C*),pH28-104b, pH28-ATPKb and pH28-A503Vb(Hsp104-A503V) are plasmids obtained from S. Lindquist lab from E. Schirmir. All the *HSP104* mutant alleles are expressed under the control of *GAL1* promoter.

The yeast two-hybrid plasmids used in chapter 4 were plasmids pAS1, which bears the *GAL4* DNA-binding domain under the *ADH* promoter and the *TRP1* marker and the plasmid pACT which bears the activation domain of *GAL4* under the *ADH* promoter and the *LEU2* marker (Durfee et al., 1993) were kindly provided by S. Elledge. Plasmids pSE1111 and pSE1112 which bear the $GAL4_{ACT}$-*SNF1* and $GAL4_{DNA}$–*SNF4* chimeric constructs respectively (Durfee et al., 1993) were used as positive control in these experiments and were also provided kindly by S. Elledge. PACTSSA1 and pACTSSB1 are SSA1 and SSB1 fused to the activation domain of *GAL4.*

Table 2.2 List and description of plasmids used in this study

Protein	Plasmid	Type/Marker	Promoter	Source[Γ]
Hsp104	pYSGAL104	CEN/*URA3*	*GAL1*	1
	pH28	CEN/*HIS3*	*GAL1*	2
Hsp104-KT-218,620	pUK21-KT218,620	CEN/*URA3*		3
	pKT218,620	CEN/*URA3*		3
	pLA1-HSP104KT	CEN/*HIS3*	*GAL1*	3
	pRS316GALHSP104KT	CEN/*URA3*	*GAL1*	3
Hsp104-MI	pYS-GAL104-MI	CEN/*URA3*	*GAL1*	1
Hsp104-MII	pYS-GAL104-MII	CEN/*URA3*	*GAL1*	1
Hsp104-MIII	pYS-GAL104-MIII	CEN/*URA3*	*GAL1*	1
Hsp101	pLA1-plantHsp104WT	CEN/*HIS3*	*GAL1*	4
Hsp101-T499I	pRS316GAL-plantHsp104MT	CEN/*HIS3*	*GAL1*	4
Hsp104-K302N	pGALHSP104-Lib	CEN/*URA3*	*GAL1*	5
Ssa1	pRS316K-SSA1	CEN/*URA3*	*SSA1*	6
	pTEF-SSA1	CEN/*URA3*	*TEF1*	6
	pGAL-SSA1	CEN/*URA3*	*GAL1*	2
	pC211	CEN/*HIS3*	*SSA2*	*7*
Ssa1-21	pRDW30	CEN/*URA3*	*SSA1*	8
Ssa2	pN2	CEN/*HIS3*	*SSA2*	7
Ssa3	pYCL1-GAL-SSA3	*CEN*/*LEU2*	*GAL1*	9
Ssa4	pYCL1-GAL-SSA3	*CEN*/*LEU2*	*GAL1*	9
	YEpGAL-SSA3	2μ/*LEU2*	*GAL1*	6
Ssb1	pRS316GAL-SSB1	CEN/*URA3*	*GAL1*	10
Ydj1	pRSYDJ1	*CEN/HIS3*	*GPD*	11
	pRS315YDJ1	*CEN/ LEU2*	*YDJ1*	12

Table 2.2 (continued)

Protein	Plasmid	Type/Marker	Promoter	Source[Γ]
Sis1	pTVSIS1	*CEN/TRP1*	*GPD*	13
	pRS315SIS1	*CEN/ LEU2*	*SIS1*	12
Human Hsp40	Hdj1424GPD	2μ/*TRP1*	*GPD*	6
Human Hsp40	Hdj2424GPD	2μ/*TRP1*	*GPD*	6
Hsp82	pMC3	*CEN/URA3*	*GAL1*	2
Hsp26	p2UGPD	2μ/*URA3*	*GPD*	14
Sla1	pFL44Sla1	2μ/*URA3*	*SLA1*	15
	pRS316GAL-SLA1	*CEN/URA3*	*GAL1*	14
Sla2	pRS316-SpSLA2	*CEN/URA3*	*SLA2*	17
	pDD355	*CEN/URA3*	*GAL1*	18
	pDD354	2μ/*HIS3*	*SLA2*	19
Sla2Δ33-359	pDD368	2μ/*HIS3*	*SLA2*	19
Sla2Δ360-575	pDD372	2μ/*HIS3*	*SLA2*	19
Ubc4	DO266	*CEN/TRP1*	*UBC4*	20
	pTRPUBC4	2μ/*TRP1*	*GAL1*	20
Ubc5	DO272	*CEN/TRP1*	*UBC4*	20
HttQ20	pAS1HUNQ20	2μ//*TRP1*	*ADH*	15
HttQ25	pYES2-25Q	2μ/*URA3*	*GAL1*	21
	pYES-Q25trp	2μ/*TRP1*	*GAL1*	22
HttQ53	pAS1HUN53	2μ//*TRP1*	*ADH*	15
HttQ103	pETQ103			5
	pYES2-103Q	2μ/*URA3*	*GAL1*	21
	pYES-Q103trp2μ/	2μ/*TRP1*	*GAL1*	22
HttPro	pYES2-PRO	2μ/*URA3*	*GAL1*	22
HttQ25Pro	pYES2-25QP	2μ/*URA3*	*GAL1*	22

Table 2.2 (continued)

Protein	Plasmid	Type/Marker	Promoter	Source[Γ]
Sup35	CEN-GAL-SUP35	*CEN/URA3*	*GAL1*	23
	pASB2	*CEN/LEU2*	*SUP35*	24
	pSTR7	2μ/ *LEU2*	*SUP35*	25
	pEMBL-yex-SUP35	2μ/ *URA3LEU2*	*SUP35*	26
	pLA1-SUP35	*CEN/HIS3*	*GAL1*	27
Sup35	pLSpSUP35NMGFP	*CEN/LEU2*	*SUP35*	28
Sup35N	pLA1-SUP35N	*CEN/HIS3*	*GAL1*	27
Sup35NM	pRS315-SpSUP35NMSc	*CEN/LEU2*	*SUP35*	*29*
	pYCL1-CUP1-SUP35NMSc	*CEN/LEU2*	*CUP1*	29
	pLSpSUP35NM-GFP	*CEN/LEU2*	*SUP35*	28
Sup35MC	pMCUP1MCSc	*CEN/URA3*	*CUP1*	5
Sup35C	pEMBL-yex-SUP35C-del3ATG	*2μ/URA3 LEU2*	*SUP35*	29

[Γ]Source indicates either reference wherever available, or the person who constructed or lab who gave gifted us the plasmid.

1: Dr Neito-Sotelo; 2: Newnam et al., 1999; 3: Wegrzyn et al., 2001; 4: Dr Elizabeth Vierling; 5: This study; 6 : Dr. E. Craig; 7: Schwimmer and Masison, 2002; 8: Jung et al., 2000; 9: Allen et al., 2004; 10: Chernoff et al., 1999; 11: Kimura et al., 1995; 12: Dr. D. Cyr; 13: Krobitsch and Lindquist, 2000; 14: Dr S.Lindquist;15: Bailleul et al., 1999; 16:Dr P. Bailleul; 17.Mike; 18: Yang et al., 1999; 19: Dr D. Drubin; 20: Dr Braun; 21: Meriin et al., 2002; 22: Dr M. Sherman; 23: Derkatch et al., 1996; 24:Borchsenius et al., 2001; 25: Broach et al., 1979; 26: Cesareni and Murray., 1987; 27: G. Newnam 28: Bailleul-Winslet et al., 2000; 29: E Lewitin 30: Ter-Avanesyan et al., 1993

pG4BD-0-SUP35N and pG4BD-SUP35 constructed by P. Bailleul (Bailleul et al., 1999) each contain *SUP35N* and *SUP35* respectively fused to the N-terminus of GAL4 DNA binding domain ($GAL4_{DNA}$). pG4BD-1-NMSc and pG4BD-1-NMPm constructed by E. Lewitin (Chernoff Lab) contains the *Saccharomyces SUP35NM* and *Pichia SUP35NM* respectively fused to the N-terminus of $GAL4_{DNA.}$ pG4BD-2-Sup35Pm constructed by P. Bailleul (Bailleul et al., 1999) contains *Pichia* Sup35 fused to the N-terminus of $GAL4_{DNA}$. Plasmids used for Ssb studies in chapter 4 were also kindly provided by Dr E. Craig. All the Ssb plasmids are *URA3* based centromeric vectors. pSSB1-V442F carries P_{SSB1}-*SSB1-V442F* . Plasmid pRS316ΔKX is the plasmid in which KpnI and XhoI sites have

Table 2.3 List of plasmids containing chimeric genes

Protein	Plasmid	Type/Marker	Promoter	Source
HttQ20Sup35MCSc	pMCUP1Q20MCSc	*CEN/URA3*	*CUP1*	This study
HttQ53Sup35MCSc	pMCUP1Q53MCSc	*CEN/URA3*	*CUP1*	This study
HttQ103Sup35MCSc	pMCUP1Q103MCSc	*CEN/URA3*	*CUP1*	This study
HumanSup35N-*Saccharomyces* Sup35MC	pMCUP1NHs400MCSc	*CEN/URA3*	*CUP1*	This study
HumanSup35NM *Saccharomyces* Sup35MC	pMCUP1NMHs400CSc	*CEN/URA3*	*CUP1*	This study
*Pichia*Sup35NM-*Saccharomyces* Sup35C	pYCLPmSc	*CEN/LEU2*	*SUP35*	*E. Lewitin*
Pichia Sup35N-*Saccharomyces Sup35MC*	pMCUP1NPmMCSc	*CEN/URA3*	*CUP1*	This study
Sup35NM-HumanHpr6.6	pRS315SpSup35NMScHPR6.6	*CEN/LEU2*	*SUP35*	*E. Lewitin*
Sup35NM-HumanHpr6.6	pYCL1CUP1Sup35NMScHPR6.6	*CEN/LEU2*	*CUP1*	*E. Lewitin*

Been destroyed in the polylinker. Plasmids -pRS316ΔKX-T433A-SSB1-X, pRS316ΔKX-F444S-SSB1-X, pRS316ΔKX-T444I-SSB1-X, pRS316ΔKX-M409A-SSB1-X, pRS316ΔKX-F432S-SSB1-X each contain *SSB1-T433A, SSB1-F444S, SSB1-T444I, SSB1- M409A and SSB1- F432 SSB* mutant alleles expressed under *SSB1* promoter (Pfund et al., 2001). The His-tagged C-terminal truncations were each carried on a p416-TEF plasmid and they are called- p416-TEF-HIS-Ssb1Δ488, p416-TEF-HIS-Ssb1Δ499 and p416-TEF-HIS-Ssb1Δ507 carrying *SSB1Δ488*, *SSB1Δ499* and *SSB1Δ507* mutant alleles expressed under *TEF* promoter (Pfund et al., 2001). pRS316K-SSB1 contains *SSB1* expressed under its own promoter and pRS316 was used as an empty vector control. The *SSA1/SSB1* chimeric gene fusions were carried on two plasmid sets. One was expressed under its own promoter and was pRS316K based while the other was

Table 2.4 List of control plasmids

Plasmid	Type/Marker	Promoter	Source
pRS316GAL	*CEN/URA3*	*GAL1*	Liu et al., 1992
pLA1	*CEN/HIS3*		Newnam et al., 1999
pEMBL-yex	*2μ/URA3 LEU2*		Cesareni and Murray, 1987
pFL39	*CEN/TRP1*		Bonneaud et al., 1991
YEP13	*2μ /LEU2*		Broach et al., 1979
pYSGAL	*CEN/URA3*		Dr Neito-Sotelo
pYCL1	*CEN/LEU2*		E. Lewitin
pFL36	*CEN/LEU2*		Chernoff Lab
pUK21			Vieira and Messing, 1991

expressed under *TEF* promoter. Both are *URA3* based centromeric vectors. Plasmid pRS316K-ABB contains the *SSA1/SSB1* chimeric gene fusion that contains the promoter and ATPase domain of *SSA1*, then the 18K peptide binding domain and the 10K variable domain of *SSB1*. Similarly, pRS316K-AAB contains the *SSA1/SSB1* chimeric gene fusion that contains the promoter, ATPase domain and 18K peptide binding domain of *SSA1*, and the 10K variable domain of *SSB1*. Plasmid pRS316K-BBA contains the *SSA1/SSB1* chimeric gene fusion that contains the promoter and ATPase domain of *SSB1*, then the 18K peptide binding domain and the 10K variable domain of *SSA1*. pRS316K-BAB contains the *SSA1/SSB1* chimeric gene fusion that contains the promoter, ATPase domain and the 10K variable domain of *SSB1* and the 18K peptide binding domain of *SSA1*, pRS316K-BAA contains the *SSA1/SSB1* chimeric gene fusion that contains the promoter and ATPase domain of *SSB1* and the 18K peptide binding and 10K variable domain domain of *SSA1*. Genes on plasmids from other set all are expressed under *TEF* promoter and the combinations are same as mentioned above for the first set. The plasmids are pTEF-BAB, pTEF-SSA1, pTEF-BAA, pTEF-BBA, pTEF-AAB ,pTEF-ABA, pRS316K-BAB, pTEF-ABB and pTEF-SSB1.

The pEMBL series vectors listed in tables 2.2 and 2.4 contain the defective *LEU2-d* allele; selection on –Leu medium leads to amplification of this plasmid (or its derivatives) up to about 100 copies per cell (Derkatch et al., 1996).

Antibodies

All primary and secondary antibodies, and the concentrations in which they were used (unless noted otherwise in corresponding chapters), are listed in Table 2.5.

Table 2.5 Description of antibodies used in this study

Antibody designation	Concentration of primary antibody for Western blotting	Concentration of secondary antibody used for Western blotting
Anti-Rnq	1:5000	1:2000
Anti-Sup35	1:1000	1:1000
Anti-GFP	1:4000	1:1000

Methods

Molecular biology techniques

Standard protocols were used for DNA electrophoresis, restriction digestion, ligation, and bacterial transformation (Sambrook and Russel, 2001). Enzymes were purchased from New England Biolabs and Gibco BRL.

QIAgen Gel Extraction protocol

Fragments of DNA generated by restriction digest or PCR reaction were separated using standard DNA electrophoresis (Sambrook and Russel, 2001). DNA bands corresponding to desired products were identified using a UV transilluminator (Fischer Biotech 312nm Variable Intensity Transilluminator) and bands were excised from EtBr-stained gels using a scalpel. Separation of DNA from gel was achieved using the QIAgen Gel Extraction Kit and protocols supplied by the manufacturer (QIAgen).

E.coli plasmid DNA isolation

Small-scale plasmid DNA isolation was performed using the QIAgen Quick Spin method or alkaline lysis method. For QIAgen Quick Spin method, protocols provided by the manufacturer (QIAgen) were followed.

For large scale/maxi prep isolation of plasmid DNA, standard laboratory protocols were applied (Sambrook and Russel, 2001). Fresh bacterial colonies were suspended in 250 ml of Luria broth (LB) (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, pH 7.0) contained in a 1 liter flask, plus an appropriate antibiotic (i.e. Ampicillin) for plasmid selection. Cells were grown to an optical density (OD) reading of 0.8 at 550 nm (OD_{550}). Cells were transferred to sterile plastic bottles, and pellets were harvested at 7000 x g. Pellets were suspended in 10 ml Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), were transferred to sterile Oak Ridge tubes, and were respun at 7000 x g. Pellets were resuspended in 4.5 ml of Solution I plus lysozyme (0.5 ml of 20mg/ml lysozyme), incubated at room temperature for 10 minutes, then were placed on ice for 20 minutes, after which 10 ml of freshly prepared Solution II (0.2N NaOH, 1% SDS) was added, and cells were returned to ice bath for an additional 15 minutes. 7.5 ml of sodium acetate (3 M, pH 5.0) was added and incubation of cells on ice continued for 1 hour. Cell debris was pelleted at 27,000 x g at 4°C for 20 minutes. Supernatants were collected into sterile tubes, and 20 ml isopropanol was added and samples were incubated for 20 minutes at room temperature, followed by 20,000 x g spin for 15 minutes, and a wash with 70% ethanol. Dried pellets were resuspended in 3.9 ml TE (pH 8.0) and were incubated for 30 minutes at 37°C, after which 4 ml LiCl (9 M) was added and the samples were transferred to -20°C for at least 20 minutes. The suspension was separated by centrifugation at 20,000 x g for 20 minutes, and supernatants were transferred to sterile tubes containing 8 ml of 95% ethanol. Tubes were placed on ice for 1 hour, followed by collection of pellets at 12,000 g for 15 minutes. DNA pellets were washed with 70% ethanol, were dried, and resuspended in TE (pH 7.4).

Yeast and *E.coli* transformation procedures

All yeast transformations were performed according to lithium-treatment procedure (Ito, et al., 1983; Kaiser, et al., 1994). All *E.coli* transformations were prepared using chemically competent *E. coli* cells according to standard laboratory protocols (Sambrook and Russel, 2001).

Standard yeast media and growth conditions

Yeast cultures were grown at 30°C unless otherwise noted. Standard yeast media and standard procedures for yeast cultivation, phenotypic and genetic analysis, transformation, sporulation and dissection were used (Kaiser, et al., 1994). Sporulating cultures were dissected using a micromanipulator Ergaval Series 10 from Carl Zeiss. Calculation of number of generations (G) for the time period t was calculated according to the following formula: $G = log_2(C_t/C_0)$, where C_t is the concentration of the cells at the time point t, and C_0 is the concentration of the cells at the starting point. Cell counts were performed using a hemacytometer (Brightline). Synthetic media lacking adenine, histidine, leucine, tryptophane, or uracil are designated as –Ade, -His, -Leu, -Trp and -Ura, respectively. In all cases when the carbon source is not specifically indicated, 2% glucose (Glu) was used. The synthetic medium containing 2% galactose (Gal) or 2% galactose and 2% raffinose (Gal+Raf) instead of glucose was used to induce *GAL* promoter. Liquid cultures were grown with at least a 1/5 liquid/flask volumetric ratio in a shaking incubator (200-250 rpm). Yeast transformants were checked in all cases on YPG (medium containing glycerol as carbon source). Petites that are respiratory deficient do not grow on YPG or on medium containing galactose as sole carbon source and were not considered for future use.

Yeast DNA isolation

Plasmid and genomic DNA from yeast cultures was collected according to standard laboratory protocols (Kaiser, et al., 1994). Briefly, cells from late log phase cultures were centrifuged at 7000 x g, and cell pellets were resuspended in 500 ul of 1M Sorbitol, 0.1 M EDTA, pH 7.5 containing 4% of a 50 ug/ml lyticase solution and were incubated at 37° C for approximately 3 hours. Cells were briefly spun down at 12,000 x g, and pellets were resuspended in 500 ul of a 50 mM Tris-HCl (ph 7.4), 20 mM EDTA solution. SDS was added to a final concentration of 1%, and the samples were incubated at 65°C for 30 minutes. 2 ml of 5 M potassium acetate was added and samples were placed on ice for 1 hour. Following 12,000 x g centrifugation, 0.75 ml isopropanol was added to the supernatants, samples were centrifuged at 12,000 x g for 5 minutes. Supernatants were discarded and pellets were dried, resuspended in 0.4 ml TE (pH 7.4) plus 22 ul of a 1 mg/ml solution of RNAse A, and incubated at 37°C for 30 minutes. DNA was precipitated with 2 volumes of 95% isopropanol. Samples were centrifuged at 12,000 x g for 15 minutes, and pellets were washed with 70% ethanol. DNA pellets were dried and resuspended in 50 ul TE (pH 7.4).

Protein isolation and differential centrifugation

Collection and analysis of yeast total protein lysates was conducted using standard yeast laboratory procedures (Kaiser, et al., 1994; Sambrook and Russel, 2001). Analysis of soluble and insoluble material of yeast total lysates ('centrifugation' or 'sedimentation' analysis) was prepared according to previously published procedures (Patino, et al., 1996), with slight modification. At least 2 independent cultures (originated from independent transformants) were analyzed per each strain/plasmid combination. For Q25/Q103 blots, cells were collected by centrifugation and disrupted by 10min

vortexing with 600 μM acid-washed glass beads in the lysis buffer (40 mM HEPES [pH7.5], 50 mM KCl, 1% Triton X-100, 1 mM Na_3VO_4, 50mM β-glycerophosphate, 50 mM NaF, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine, and 0.2 of one proteinase inhibitor tablet, Roche, pre-dissolved in distilled water). Cell debris were removed by centrifugation at 800 g for 10 min. This step also precipitates some of Q103 and Q25 protein; fraction of protein precipitated in these conditions was not influenced by the chaperone composition (not shown). The resulting supernatant was fractionated by centrifugation at 10,000 g for 10 min. The pellets were dissolved in the same volume of lysis buffer as respective supernatants. Loading SDS-PAGE buffer with 2% SDS, was added to both pellets and supernatants. Samples were denatured by boiling for 5 min, ran on SDS-PAGE, transferred to nitrocellulose membrane and reacted to anti-GFP monoclonal antibody (Sigma), and the to the secondary antimouse antibody (Sigma). Horse radish peroxidase tagged to secondary antibody was detected using Amersham ECL detection system. Densitometry was performed on X-ray films by using the Kodak 1D program from a Gel Logic 200 imaging system.

For Sup35 and Rnq1 blots, yeast cells were suspended in centrifugation lysis buffer with slightly different composition (50 mM tris-HCl, pH7.5 + 10 mM KCl + 5 mM $MgCl_2$ + 0.1 mM EDTA + 0.1 mM DTT + 100μg/ml cycloheximide + 2 mM PMSF + 1 mM Benzamidine + 2μg/ml Pepstastin A + 10μg/ml Leupeptin + 100μg/ml RNase A) were lysed with glass beads on a vortex mixer. High concentrations of proteinase inhibitors were required to keep proteins stable throughout the fractionation procedure. Cell debris was removed by centrifugation at 3,000 x g to produce a “total lysate” fraction. Half of the total lysate was used as a control, while the remainder was fractionated by centrifugation at 12,000 x g for 15 min. The supernatant was placed into a fresh tube, and the pellet was resuspended in an equal amount of the lysis buffer. SDS, glycerol, 2-

mercaptoethanol and Tris-HCl (pH 6.8) were added to every sample up to final concentrations of 3%, 10%, 3% and 0.15 M, respectively. Resulting samples were heated at 95^{o}C for 10 min and run on the standard SDS-polyacrylamide gel. For performing the protein assays, gels were transferred onto Hybond ECL nitrocellulose membranes and reacted to the appropriate antibodies. Where required, densitometry measurements were obtained from exposed films using the program Alphaimager 2000, Alpha Innotech Corporation.

Nonsense suppression assay for presence of [PSI^{+}]

Presence of [PSI^{+}] was detected by its ability to suppress the *ade1-14*$_{UGA}$ mutant allele, as described previously (Chernoff, et al., 2000; Derkatch, et al., 1996). The [psi^{-}] *ade1-14*$_{UGA}$ strains are not able to grow on medium lacking adenine (-Ade) and exhibit dark red color on rich (YPD) medium, while [PSI^{+}] *ade1-14*$_{UGA}$ strains are able to grow on –Ade after 2-3 days ('strong' [*PSI*$^{+}$]) or 7-10 days ('weak' [*PSI*$^{+}$]), and exhibit light-pink color on YPD. 'Mosaic' colonies containing both [*PSI*$^{+}$] and [*psi*$^{-}$] cells were detected as sectored pink/red colonies on YPD medium. In quantitative analyses, mosaic/sectored colonies were scored as ½ [*PSI*$^{+}$] since when the cell was plated, [*PSI*$^{+}$] was present, but was unstable in successive cell divisions.

Assay to monitor for presence of [RNQ^{+}]

The non-Mendelian element [PIN^{+}] controls the ability of overproduced Sup35 to *induce de novo* formation of [PSI^{+}] (Derkatch, et al., 1997). In all of the yeast strains used in this study, [PIN^{+}] is known to be encoded by the prion form of the Rnq1 protein, designated [RNQ^{+}]. The presence of [RNQ^{+}] was monitored by one of the following assays depending on the strains used: 1) [psi^{-}] strains, bearing the galactose-inducible plasmid pLA1-SUP35, were incubated on –His/Gal medium and velveteen replica plated

onto –Ade/Glu media. [RNQ$^+$] was detected by growth on –Ade after 10-14 days of incubation, due to induction of [PSI$^+$]. 2) [psi$^-$] strains lacking pLA1-SUP35 were mated to the strain [psi$^-$ rnq$^-$] GT234 bearing the multicopy *SUP35* plasmid pSTR7 (or YEp13 empty vector control, for comparison). The [RNQ$^+$] diploids, in contrast to [rnq$^-$] diploids, grew on medium lacking adenine after 10-14 days of incubation due to induction of [PSI$^+$]. 3) To check the [PSI$^+$] strains for the presence of [RNQ$^+$], cells were first cured of [PSI$^+$] by galactose-inducible wild-type Hsp104 (Chernoff, et al., 1995), which does not cure yeast cells of [RNQ$^+$] (Derkatch, et al., 1997). At least two independently cured [psi$^-$] derivatives were usually analyzed for each [PSI$^+$] clone, always with the same result. These [psi$^-$] derivatives were either transformed individually with the multicopy *SUP35* plasmid pSTR7 (or YEp13 empty vector control, for comparison), in case of diploid strains, or mated to the [psi$^-$ rnq$^-$] GT234 bearing pSTR7 (or YEp13 empty vector control, for comparison), in case of haploid strains. Presence of [RNQ$^+$] in the resulting transformants or diploids was detected as described above.

Thermotolerance assay

To measure thermotolerance of the exponential yeast cells, yeast precultures were grown overnight at 25°C in the synthetic medium selective for the plasmid(s) containing inducer if the constructs were inducible, at a starting concentration of 2.4 x 10^5 cells/ml till final concentration was 10^6 cells/ml was reached. The cultures typically took 24hrs in shaker conditions to attain this concentration. 0.5ml aliquots of each culture were placed on ice (control samples) or incubated for 5 to 20 mins in the 50°C waterbath and then on ice (heat-shocked samples). Then serial dilutions of both the control and heat-shocked samples were prepared and either pipetted onto the corresponding synthetic selective medium containing glucose, 5μl per spot or 3μl per

spot on YPD medium. Plates were scored were scored after 3 to 4 days of incubation at 30°C.

Plate assay for cytotoxicity

To assess cytotoxicity qualitatively, yeast transformants were patched on to the Glu medium selective for the plasmid(s) and velveteen replica plated onto the inducer containing medium of the same composition eg: in order to induce the P_{GAL} promoter-galactose containing medium, to induce P_{CUP1}- $CuSO_4$ containing medium. This was followed by the second passage on inducer medium, by using either velveteen replica plating or spotting with a spotter from Replicatech if necessary. Differences in growth were better seen sometimes after the second passage. Atleast 8 independent transformants were tested in each case for each strain/plasmid combination. All or vast majority of them did usually show the same results. Typical representatives are shown on figures.

Quantitative assay for cytotoxicity

To measure cytotoxicity quantitatively, yeast cultures were pre-grown overnight in the synthetic Glu liquid medium, selective for the plasmid(s), and inoculated at 10^5 cells/ml into the liquid inducer medium eg: Gal+Raf medium of the same composition, in order to induce the P_{GAL} constructs. Aliquotes were taken after various periods of incubation, and plated onto the Glu medium selective for plasmid(s). Colonies were counted after 3 days of incubation at 30°C. Two platings for each of two independent cultures for each strain/plasmid combination were performed at each time point.

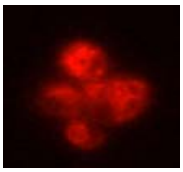
Averages of four counts are shown on Figures. Standard deviations never exceeded 35% of the value for each time-point.

GFP detection by fluorescence microscopy

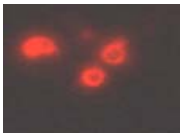
GFP fluorescence images were taken using live cultures. An aliquote of cells was placed onto a glass slide, it was sealed with a coverslip using nail polish. Images were scanned using Olympus BX41 microscope with a 100X objective, with Narrow band GFP filter. Typically, Cultures were grown overnight in the inducer containing medium eg Gal+Raf medium for genes expressed under galactose inducible promoter, as described above for quantitative analysis. Only cells showing fluorescence were counted and grouped into different classes based on the patterns observed. The excitation wavelength was 543 nm for the helium-neon laser (rhodamine fluorescence), and 488 nm for the argon laser used to visualize GFP.

Endocytosis assay

For staining with FM4-64 (Molecular Probes), mid-log phase cells grown in the Gal+Raf medium were concentrated 10-times and incubated with 8μM dye for 12 mins in the selective medium with galactose. Cells were washed two-times with the same medium and left for 45 min at 30°C shaker (Meriin et al., 2003). FM4-64 staining was detected at the BX41 fluorescence microscope with TRITC filter (see Figure 2.1).



12 minute staining



45 minute staining

Figure 2.1 Internalization of the lipophilic dye FM4-64 in normal cells The dye binds the cell membrane and is internalized through the endocytic pathway. Red patches represent the labeled endosomes after 10-15 minutes of staining and distinct ring-shaped labeling of the vacuoles after 45 minutes of staining reflecting the fusion of endosomes with vacuolar membrane is an indication that endocytosis is not affected.

CHAPTER 3

EFFECTS OF CHAPERONES ON Q - RICH PROTEIN AGGREGATES -PART I

Background

Hsp100

The ClpB/Hsp100 proteins are conserved members of the AAA^+ superfamily (Wickner et al., 1999). The AAA^+ proteins are ATPases associated with various cellular activities like protein folding, membrane trafficking, organelle biogenesis, proteolysis, intracellular motitility and DNA replication (Neuwald et al., 1999; Vale, 2000; Ogura and Wilkinson, 2001). This group of proteins is found in bacteria, protozoa, fungi and plants and their expression is inducible by heat stress (Schirmer et al., 1996). In yeast, Arabidopsis and maize, the Hsp100/ClpB play a major role in acquisition of thermotolerance ie the ability to survive very high temperatures (Sanchez and Lindquist, 1990; Queitsch et al., 2000; Nieto-Sotelo et al., 2002). Hsp104 (Yeast Hsp100) is also important in prion maintenance (Chernoff et al., 1995; Patino et al., 1996; Paushkin et al., 1996; DebBurman et al., 1997; Moriyama et al., 2000) and was shown to be essential for the aggregation of poly-Q expanded exon 1 of Htt (Q103) (Krobitsch and Lindquist, 2000; Cao et al., 2001; Kimura et al., 2001; Satyal et al., 2000).

Two models have been proposed to explain the role of Hsp104 in prion life-cycle. The first model suggests that Hsp104 converts the cellular Sup35 protein into a partially unfolded intermediate, which serves as a substrate for prion conversion (Chernoff et al., 1995). The second model suggests that Hsp104 facilitates prion conversion by combining partially unfolded intermediates into oligomeric complexes thus producing new prion ‘seeds’ initiating new rounds of prion replication (Patino et al., 1996; Serio and Lindquist, 2000).

A non-mutagenic chemical agent (GuHCl) cures all three yeast prions [PSI^+], [URE3] (Wickner, 1994) and [RNQ^+] (Derkatch et al., 1997). [PSI^+] loss by GuHCl occurs in a strictly generation-dependent manner apparently by a " dilution mechanism" blocking [PSI^+] proliferation (Tuite et al., 1981; Eaglestone et al., 2000). GuHCl is a protein-denaturing agent, however at concentrations used (1-5mM), it is not high enough to cause significant protein denaturing. These concentrations however have been shown to inhibit ATPase activity of Hsp104 in vitro (Glover and Lindquist, 1998). This suggests that GuHCl cures yeast cells of prions by inactivating Hsp104 (Eaglestone et al., 2000; Glover and Lindquist, 1998; Ness et al., 2002; Ferreira et al., 2001; Jung et al., 2002). This suggestion is further supported by the finding that a point mutation at residue 184 prevents [PSI^+] curing by GuHCl, implicating Hsp104 as the major target of GuHCl in prion replication cycle (Jung et al., 2000).

The functional unit of the Hsp104 is composed of six monomers each with two ATP binding sites (nucleotide binding domains one and two also known as NBD1 and NBD2). The NBDs are highly conserved (Schirmer et al., 1996) flanked by less conserved amino-terminal, middle, and carboxy-terminal regions (Parsell et al., 1991). In related proteins, the N and C-terminal regions appear to bind substrates and/or effector proteins (Smith et al., 1999; Barnett et al., 2000). The middle region is the most variable and least understood. Even though the middle domain is not conserved, its length and the primary and secondary structure has been conserved during evolution (Nieto-Sotelo et al., 1999). The middle region has a propensity to form a coiled-coil (Nieto-Sotelo et al., 1999). Both NBDs are essential for function: substitution of critical lysine residues at position 218 and 620 in the Walker-A sites by threonine in either NBD eliminates thermotolerance (Parsell et al., 1994) and interferes with prion maintenance function (Chernoff et al., 1995). Careful analysis indicated that the single mutation at position 218 (NBD1) prevents hydrolysis of ATP, not affecting the oligomerization of Hsp104, while

the 620 mutation impairs Hsp104 oligomerization, but not ATP hydrolysis (Schirmer et al., 1998; Parsell et al., 1994). The Hsp104-KT mutants are dominant-negative for [PSI^+] curing (Chernoff et al., 1995) and thermotolerance (Wegrzyn et al., 2001). The Hsp104-KT mutant also cures [PIN^+] (Wegrzyn et al., 2001). A great degree of conservation in the nucleotide-binding domain and middle domain amongst plant, yeast and bacterial orthologs is seen. Plant Hsp101 shares 60% similarity with Yeast Hsp104. Moreover, expression of Hsp101 could complement the thermotolerance defect in yeast caused by deletion of *HSP104* .

Ssa proteins –members of the Hsp70 family are also coinduced with Hsp104 during heat shock, stationary-phase growth and sporulation (Kurtz et al., 1986; Werner-Washburne and Craig, 1989). They interact with Hsp104 in thermotolerance in vivo (Sanchez et al., 1993) and in protein disaggregation in vivo (Glover and Lindquist, 1998). Ssa1 favors [PSI^+] propagation. Hsp104's role is to cause aggregate disassembly, and Hsp70-Ssa is proposed to promote new rounds of aggregate assembly. This concerted action of Hsp104 and Hsp70-Ssa results in prion proliferation.

In this chapter, we present a detailed study of the effects of Hsp104-wild-type and different mutants (NBD, Middle domain and C-terminal region) including plant Hsp104 (Hsp101) on Q-rich aggregates formed by Sup35, Rnq1 and Htt-Q103. We also present kinetics of [PSI^+] and [PIN^+] loss by Hsp104, Hsp104-KT and GuHCl.

Materials and methods

Yeast Strains

Yeast strains used in this study are discussed in detail in Chapter 2 and Table 2.1. The [PSI^+ pin^-] strain GT490 was OT56, cured of [PIN^+] by expressing the dominant negative Hsp104 derivative Hsp104-KT218,620 (Wegrzyn, et al., 2001). For this

purpose, OT56-derived colonies that retained [PSI$^+$] after pulse induction of Hsp104-KT218,620 expression, were then cured of [PSI$^+$] by excess wild-type Hsp104 and checked for retention of [PIN$^+$] (see description of the [PIN$^+$] assay in Chapter 2 Materials and Methods section). As wild-type Hsp104 usually does not cure [PIN$^+$], those becoming [pin$^-$] after excess Hsp104 treatment were identified as originating from the [PSI$^+$ pin$^-$] derivatives. Lack of the aggregated (prion) form of Rnq1 in these derivatives was then confirmed by differential centrifugation assay (Chernoff, et al., 2002).

Plasmid constructs

Plasmids specific to this study are described here, for all other commonly used plasmids refer to Chapter 2. The plasmid pUK21-KT218,620 was constructed by inserting XhoI-EcoRI fragment of *HSP104* obtained from plasmid pKT218,620 into XhoI-EcoRI digested plasmid pUK21 (Vieira and Messing, 1991). The plasmid pKT218,620 provided by S. Lindquist contains two Lys-to-Thr substitutions at codons 218 and 620 of the *HSP104* gene that inactivate both the ATP binding sites of Hsp104 (Parsell et al., 1991). The *HIS3*-based plasmid pLA1-HSP104KT and *URA3*-based plasmid pRS316GAL-HSP104KT which express the *HSP104KT218,620* mutant allele under the *GAL* promoter, were constructed by inserting the 3.2kb BamH1-SpeI fragment of mutant Hsp104 from pUK21-KT218,620 into BamH1-SpeI digested pLA1 and pRS316GAL respectively (Wegrzyn et al., 2001).

Dr. Elizabeth Vierling's lab kindly provided us the plasmid pAZ958. The BamHI-XbaI fragment contains *HSP101* cDNA (wild type) from Arabidopsis and plasmid pAZ1092 BamHI-XbaI fragment contains mutant *HSP101* (hot1-4) from Arabidopsis. The change is a G to an A at position 1495. These fragments were cloned into yeast expression vectors in order to study them in yeast. The *HIS3*-based plasmid pLA1-plantHsp104WT that expresses Wild-type Plant Hsp101 under the *GAL* promoter was

constructed by inserting the BamH1-Xba1 *HSP101* fragment from plasmid pAZ98 into BamH1-Xba1 digested pLA1. Similarly, pLA1-plantHsp104MT was constructed by inserting BamH1-Xba1 Mutant *HSP101* fragment from plasmid pAZ1092 into BamH1-Xba1 digested pLA1.

Quantitation of prion curing

Transformants were grown in the same manner as described in materials and methods- quantitative assay section. Growth was monitored by counting cells and cultures were maintained in the exponential phase of growth by periodically diluting them with fresh medium. Aliquots were taken after certain periods of time and were plated onto synthetic plasmid-selective Glu medium. Colonies were counted and analyzed for the presence of [PSI^+] and [PIN^+] as described in Chapter 2.In addition to complete [PSI^+] and [psi^-] colonies, a fraction of the mosaic colonies that showed both light pink [PSI^+] and red [psi^-] sectoring were counted as half [PSI^+] and half [psi^-]. The number of generations (G) for the time period t was calculated according to the formula : $G = \log_2 (C_t / C_0)$ where C_t is the concentration of viable plasmid-containing cells at time-point t and C_0 is the concentration of the viable plasmid-containing cells at the starting point. Concentrations of viable plasmid-containing cells were determined by from the numbers of colonies grown on selective medium. Curing by 5mM GuHCl was quantified in exactly the same way except that the strains did not contain galactose-inducible plasmids, both cultures growing in synthetic Gal + Raf medium and in YPD medium were analyzed for comparison, aliquots were plated onto YPD medium, and all viable cells rather than only plasmid-containing cells were counted to calculate the numbers of generations.

Results

The data on Hsp104 and Ssa effects on [PSI$^+$] is published in Wegrzyn et al., 2001. The data on Hsp104 effects on poly-Q toxicity has been submitted for publication to the Journal of Biological Chemistry as a part of Gokhale et al., 2005 manuscript.

Comparison of the kinetic parameters of prion curing by excess Hsp104 Wild-type, mutant Hsp104-KT and GuHCl

GuHCl, an agent that blocks [PSI$^+$] proliferation (Eaglestone et al., 2000) presumably does so by inactivating Hsp104 (Eaglestone et al., 2000; Glover and Lindquist, 1998). To check this, we compared kinetics of [PSI$^+$] loss by wild-type Hsp104, mutant Hsp104-KT and GuHCl in the [PSI$^+$PIN$^+$] strains GT81-1C (Figure 3.1 A), OT55 (Figure 3.1 B) and OT56 (Figure 3.1 C). As wild-type and mutant Hsp104 were induced in synthetic Gal + Raf medium, the GuHCl induced curing of [PSI$^+$] was also studied in both YPD and Gal + Raf medium. Although GT81-1C and OT56 were more resistant to [PSI$^+$] curing effect of GuHCl in Gal + Raf medium than in YPD medium, it did not change the general picture. In all cases, no statistically significant [PSI$^+$] loss was observed for the first 4 to 5 (strong [PSI$^+$] cultures- Figure 3.1 A, C) or 3 to 4 (weak [PSI$^+$] culture-Figure 3.1 B) cell divisions in the presence of GuHCl, confirming the existence of a lag period apparently required for dilution of [PSI$^+$] replicating units. In contrast cultures overexpressing either Wild-type or mutant Hsp104 did not exhibit this lag. The weak [PSI$^+$] strain OT55 was more sensitive to the curing effect of wild-type Hsp104 but not to the curing effects of mutant Hsp104 than was the isogenic strong [PSI$^+$] strain OT56 (Figure 3.1 B, C). This further confirms a difference between the mechanisms of [PSI$^+$] curing effects of wild-type Hsp104 and mutant Hsp104-KT. Quantitation of [PIN$^+$] curing in the [psi$^-$ PIN$^+$] strain OT60 demonstrates that [PIN$^+$] loss by GAL-HSP104KT is very

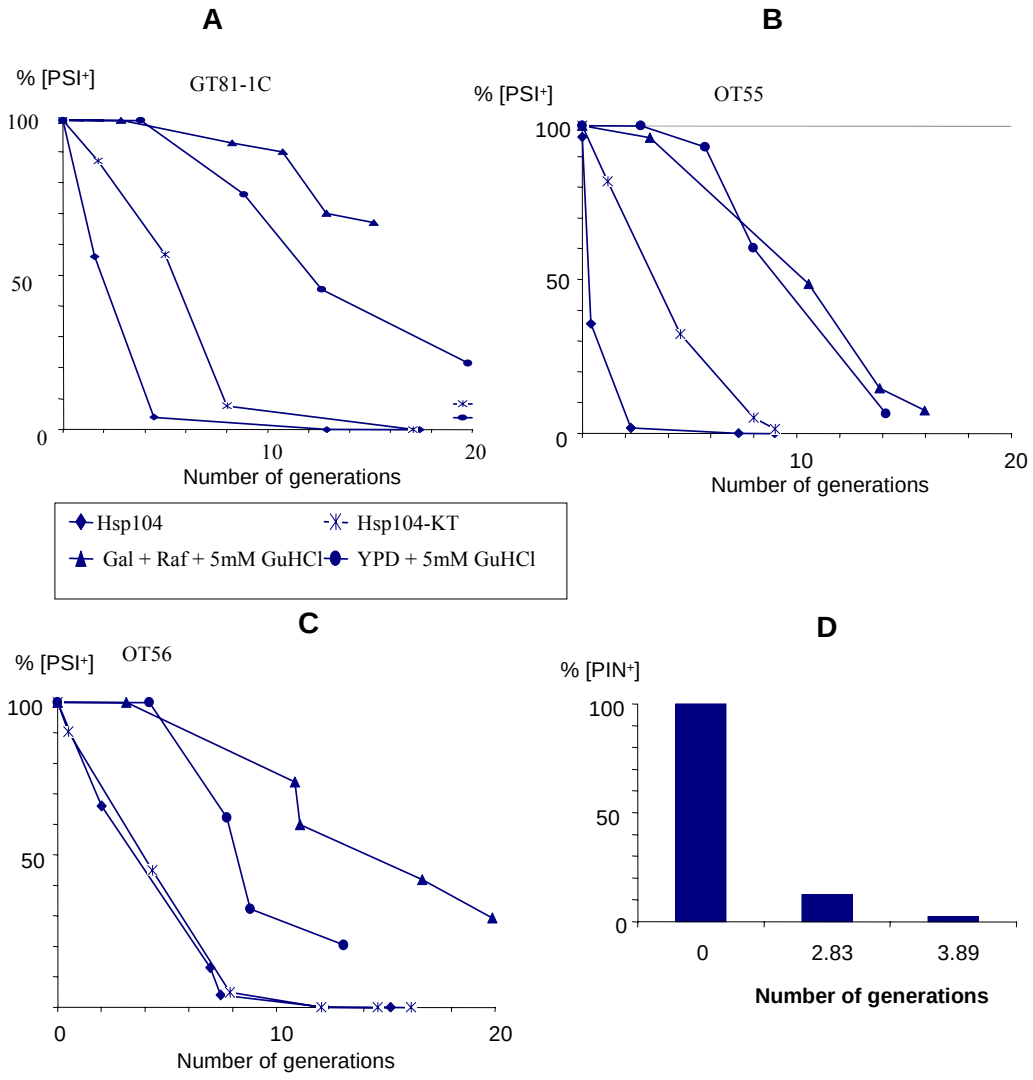


Figure 3.1 Kinetics of [PSI$^+$] and [PIN$^+$] loss
A, B and C. Comparison of kinetic parameters of [PSI$^+$] curing by WT Hsp104, Hsp104-KT and GuHCl in the yeast strains A. GT81-1C , B. OT55 and C. OT56. Curing conditions included induction of *GAL-HSP104* (Hsp104), induction of *GAL-HSP104-KT* (Hsp104-KT), growth in the presence of 5 mM GuHCl in rich medium (YPD + 5 mM GuHCl) or in synthetic Gal + Raf medium (Gal + Raf + 5mM GuHCl). See materials and methods for procedures. D. Quantitation of [PIN$^+$] curing in the [psi$^-$ PIN$^+$] OT60 by Hsp104-KT. Expression of *GAL-HSP104KT* was induced on –Ura/Gal medium. Cells were plated onto –Ura medium after various periods of time. Presence of [PIN$^+$] in resulting colonies was determined in a cross to the tester strain GT234/pSTR7 as described in Materials and Methods. [PIN$^+$] curing is very rapid and complete by third generation.

Table 3.1 Distribution of [PIN$^+$] among [PSI$^+$] and [psi$^-$] colonies obtained after excess Hsp104 or excess Hsp104-KT treatment

Strain	Induced construct	No. of Generations	Distribution of [PSI$^+$]		Distribution of [psi$^-$]	
			No. (%) [PIN$^+$]	Total no of [PSI$^+$] analyzed	No. (%) [PIN$^+$]	Total no of [PSI$^+$] analyzed
OT55	*GAL-HSP104*	0.4	NT		8 (100)	8
		2.3	8 (100)	8	8 (100)	8
		7.3	NT		8 (100)	8
OT55	*GAL-HSP104KT*	1.2	16(80)	20	1 (33)	3
		4.0	2(8)	24	1 (6)	16
		8.0	NT		0	3
		9.0	NT		0	5
OT56	*GAL-HSP104*	2.0	8 (100)	8	8 (100)	8
		7.0	NT		8 (100)	8
		7.5	NT		8 (100)	8
OT56	*GAL-HSP104KT*	0.5	NT		2 (29)	7
		4.3	5 (21)	24	0	26
		7.9	5 (21)	24	0	21

NT: Not tested.

rapid and is nearly complete by third generation (Figure 3.1 D). The [pin$^-$] cells were detected as early as in the first generation after Hsp104-KT induction (Table 3.1). Quite remarkably [PIN$^+$] appeared to be more frequently retained in cells remaining [PSI$^+$] than in cells becoming [psi$^-$]; in strain OT56, [PIN$^+$] was still retained by some [PSI$^+$] cells after eight generations, yet it was entirely lost from [psi$^-$] cells after four generations (Table 3.1). This is in agreement with preferential coretention and coloss of [PSI$^+$] and [PIN$^+$] after GuHCl treatment that was reported previously (Derkatch et al., 2000).

Mutant Hsp104-KT interferes with the activity of wild-type Hsp104

One possible mechanism of Hsp104-KT to cure [PSI$^+$] is by interfering with Wild-type Hsp104 by inhibiting assembly of functional Hsp104 hexamers. That is indeed the case with K620T as it impairs Hsp104 oligomerization in vitro. It is likely that in vivo, the interaction between mutant and wild-type Hsp104 leads to the formation of heteromultimeric complexes so that the formation of functional Hsp104 hexamers becomes inefficient. If so, this effect should be partly overcome by increasing concentration of wild-type Hsp104. Similarly, Hsp104-KT show partly ameliorate the [PSI$^+$] curing effect of excess Wt Hsp104. To check this, we overexpressed *GAL-HSP104* and *GAL-HSP104KT* constructs separately and together in [PSI$^+$] strain OT56. [PSI$^+$] curing by wild-type or mutant Hsp104 alone proceeded with similar rapid kinetics such that less than 60% of cells remained [PSI$^+$] one generation after galactose induction and less than 10% of cells remained [PSI$^+$] after four generations (Figure 3.2 A). In contrast, more than 80% of cells remained [PSI$^+$] after one generation and more than 40% remained [PSI$^+$] after 4 generations when both mutant and wild-type were expressed together (Figure 3.2 A). This indicates that wild-type and mutant Hsp104 interfere with each other's [PSI$^+$] curing effects as would be expected if Hsp104-KT acts by inactivating wild-type Hsp104.

Interactions of Hsp70-Ssa with wild-type and mutant Hsp104

Hsp70-Ssa protects [PSI$^+$] curing by excess wild-type *Hsp104* (Newnam et al., 1999). In order to examine whether it does so with mutant Hsp104-KT, we expressed *GAL-SSA1* construct with either wild-type *HSP104* or mutant *HSP104-KT* in the [PSI$^+$] strain OT56. Hsp70-Ssa did not protect [PSI$^+$] curing by excess mutant Hsp104-KT (Figure 3.2 B). Moreover, [PSI$^+$] curing by Hsp104-KT appeared to be slightly more

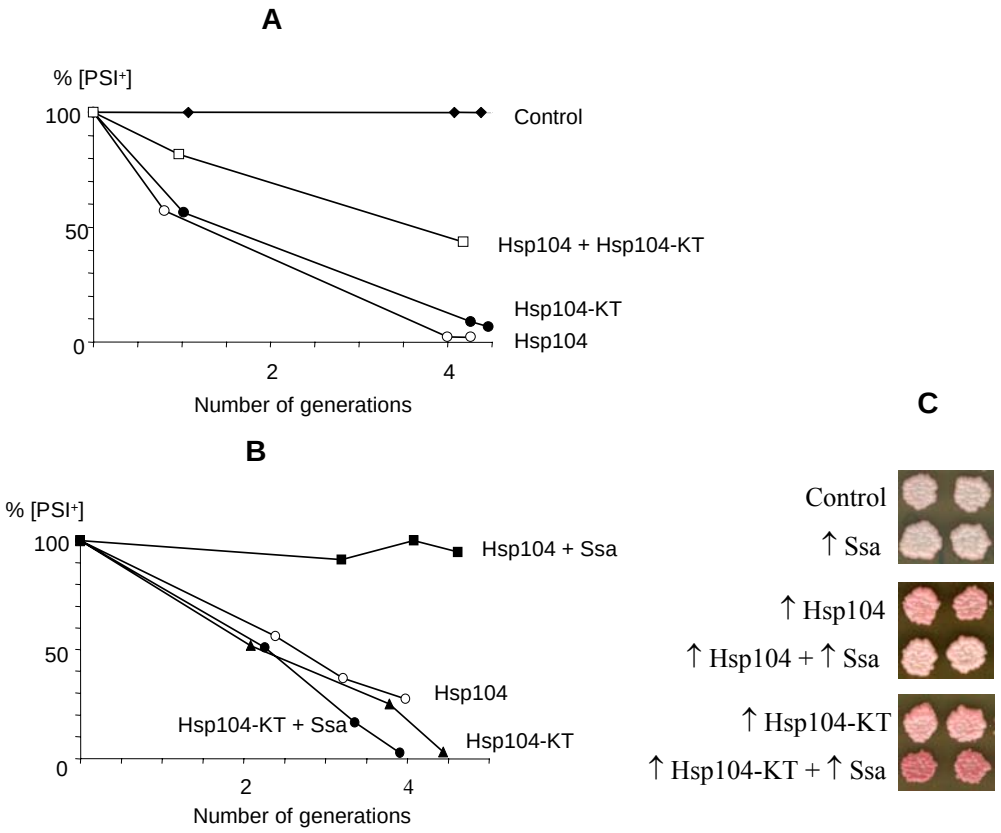


Figure 3.2 Interactions of wild-type Hsp104, mutant Hsp104-KT and Hsp70-Ssa A. Antagonistic interaction between mutant and wild-type Hsp104 in [PSI+] curing. Yeast strain was transformed with pH28 (Hsp104), pLA1-HSP104KT (Hsp104-KT), a combination of both plasmids or controls. Cultures were induced in liquid –Ura-His/Gal + Raf medium (see materials and methods) and plated onto YPD medium at various time points to detect [PSI+] curing. Both wild-type and mutant Hsp104 proteins cure [PSI+] completely by fourth generation of growth. Coexpression of wild-type and mutant Hsp104 reduces [PSI+] curing. B. Effects of Hsp104 and Ssa on [PSI+] curing Yeast strain was transformed with plasmids pH28 + pRS316GAL (Hsp104), pH28 + pGAL-SSA1 (Hsp104 + Ssa), pLA1-HSP104KT + pRS316GAL (Hsp104-KT) and pLA1-HSP104KT + pGAL-SSA1(Hsp104KT + Ssa). Cultures were induced in liquid –Ura-His/Gal + Raf medium. Excess Hsp70-Ssa protects [PSI+] from curing by excess Hsp104 but not from excess Hsp104-KT. C. Effects of Hsp70-Ssa on [PSI+] curing by color assay. In addition to the plasmids used in legend 4.2B , were combinations pGAL-SSA1(Ssa1) and pLA1 (control). Transformants were grown on solid –Ura-His/Gal medium and velveteen replica plated onto YPD medium. Lighter color is indicative of intense suppression. Results confirm that excess Hsp70-Ssa decreases the [PSI+] curing effect of excess Hsp104 but increases the PSI+] curing effect of excess Hsp104-KT
Yeast strain used: OT56 [PSI+ PIN+]

efficient in the presence of excess Ssa. Yeast cultures expressing Hsp70-Ssa and wild-type Hsp104 simultaneously exhibited lighter color on YPD medium compared to cultures overexpressing Hsp104 alone. This corresponds to higher retention of [PSI$^+$] mediated nonsense suppression in the presence of excess Hsp70-Ssa. However efficiency of [PSI$^+$] mediated nonsense suppression was reduced in cultures that underwent simultaneous overexpression of Hsp70-Ssa and Mutant Hsp104KT compared to that of the cultures expressing Hsp104-KT alone. This reduction resulted in a more intense red color on YPD medium (Figure 3.2 C) Thus Hsp70-Ssa exhibits opposite effects on [PSI$^+$] curing by overproduced wild-type and mutant Hsp104.

Effects of the mutant alleles of yeast Hsp104 and plant Hsp101 on [PSI$^+$] and [PIN$^+$]

Overproduction of the yeast chaperone Hsp104 is known to eliminate [PSI$^+$] (Chernoff et al., 1995) but not [PIN$^+$] (Derkatch et al., 1997). The double K-to-T substitution at aa positions 218 and 620 of Hsp104 (Hsp104-KT) inactivates both nucleotide binding domains of Hsp104 (NBD1 and NBD2) and disrupts the function of wild-type Hsp104 in a dominant negative fashion (Chernoff et al., 1995; Schirmer et al, 1998). In contrast to overproduced wild-type Hsp104, mutant Hsp104-KT cures yeast cells not only of [PSI$^+$] (Chernoff et al., 1995) but also of [PIN$^+$] (Wegrzyn et al., 2001). Hsp104-K302N (refer below for the description of how K302N was isolated) has a mutation in its nucleotide binding domain. It cures both [PSI$^+$] and [PIN$^+$] prions (Table 3.2).

Next, we checked if Hsp104 mutants previously isolated in a genetic screen at S. Lindquist laboratory (Schirmer et al., 2004) affect [PSI$^+$] and [PIN$^+$] (see Table 3.2). Some of the mutations were in the nucleotide binding domain (Hsp104-NBDDA) –cured [PSI$^+$] (Table 3.2) while some in the middle domain (Hsp104-A503V, Hsp104-A503VA509D and Hsp104-A503V) did not cure [PSI$^+$] and [PIN$^+$] except for Hsp104-

A509D (Table 3.2) and C-terminal domain (Hsp104-C* where C* indicates several substitutions from negatively charged residues to positively charged ones) of Hsp104did not cure [PSI$^+$] and [PIN$^+$](Table 3.2). Some mutant alleles had mutations in more than

Table 3.2. Effects of different Hsps on yeast prions

Overexpressor combinations	**Tested for [PSI$^+$]**			**Tested for [PIN$^+$]**		
	[PSI$^+$]	**[psi$^-$]**	**Total**	**[PIN$^+$]**	**[pin$^-$]**	**Total**
Control	48	0	48	48	0	48
↑ WT Hsp104	0	48	48	48	0	48
↑ Library Hsp104 (Hsp104-K302N)	0	48	48	0	48	48
↑ Hsp104-A509D	21	27	48	18	30	48
↑ Hsp104-A503V	Not applicable*			48	0	48
↑ Hsp104-A503VA509D	45	3	48	48	0	48
↑ Hsp104-NBDDA	0	48	48	Not tested		
↑ Hsp104-Internal deletion at C-terminal	3	45	48	45	3	48
↑ Hsp104-C*	36	12	48	48	0	48
↑ Hsp104-G217ST499I	0	48	48	Not tested		
↑ Hsp104-A392TT499I P679KK620T	5	43	48	45	0	48
↑ Hsp104- L421KI424DI428K (MI)	15	33	48	48	0	48
↑ Hsp104- L445RA448DL455K (MII)	14	34	48	0	48	48
↑ Hsp104- A479DL483KL486R (MIII)			Not applicable$^\delta$			
↑ Plant WT Hsp104	48	0	48	48	0	48
↑ Plant Hsp104-A499T	48	0	48	48	0	48

* Hsp104-A503V is toxic to [PSI$^+$]

$^\delta$ Hsp104- A479DL483KL486R (MIII) is toxic to [PSI$^+$] and [PSI$^-$]

one domain eg Hsp104-G21ST499I –cured [PSI$^+$] and Hsp104-A392TT499IP679KK620T cured [PSI$^+$] but not [PIN$^+$] while one mutant allele had internal deletion in C-terminal domain that also cured [PSI$^+$] but not [PIN$^+$] (Table 3.2). Hsp104-A503V by itself was toxic to the [PSI$^+$] strain (see in more detail below) and did not cure

[PIN$^+$] (Table 3.2) The Hsp104-A503V derivative was also previously shown not to antagonize thermotolerance (Schirmer et al., 2004).

Amongst the mutant alleles of Hsp104 with triple aminoacid substitutions in amphipathic α-helices of middle domain that has a propensity to form a coiled-coil (Schirmer et al., 1996), MI (mutations affecting helix 1) and MII (mutations affecting helix 2) both cured yeast prion [PSI$^+$] though not completely, with only MII curing [PIN$^+$] as well (Table 3.2). MIII (mutations affecting helix 3) remains toxic in the strain background tested. Mutations that changed the amphipathic character on helices 2 (MII) and 3 (MIII), but not those on helix 1(MI) caused the loss of function of Hsp104 suggesting that helices 2 and 3 play an important role in the maintenance of a coiled-coil structure. Moreover, these mutations abolished the hexamer formation and changed the protein stability (Neito-Sotelo et al., unpublished results).

Plant Hsp101 shares the domain architecture of middle domain with the yeast Hsp104. As in yeast, Hsp101 plays a major role in the acquisition of thermotolerance ie the ability to survive high temperatures, following acclimation to mild heat shock treatments (Sanchez and Lindquist, 1990; Queitsch et al., 2000; Nieto-Sotelo et al., 2002). The T499A mutation falls in the coiled-coil linker between the two NBDs and corresponds to yeast position 502. This mutant failed to develop thermotolerance to 45°C providing an evidence for an essential role of this structural domain. When checked for their effects on yeast prions, both Wild-type and mutant Plant Hsp104 did not cure either [PSI$^+$] or [PIN$^+$] (Table 3.2). In all cases, 12 subclones from 4 independent transformants were checked for [PSI$^+$] and [PIN$^+$] as given in materials and methods, Chapter 2.

[PSI$^+$]-specific toxicity of Hsp104-A503V

As mentioned above, expression of Hsp104-A503V by itself inhibited growth of the [PSI$^+$ PIN$^+$] strain OT56 and [PSI$^+$ pin$^-$] strain GT490. Such an effect was not seen in the isogenic [psi$^-$ PIN$^+$] strain OT60, confirming that it is specific to [PSI$^+$] rather than to any prion (Figure 3.3 A, B). The severity of the toxic effect of Hsp104-A503V depended on the variant of [PSI$^+$]. The [PSI$^+$ PIN$^+$] strain OT55, which is isogenic to OT56 but bears a "weak" variant of [PSI$^+$], characterized by the larger proportion of the Sup35 protein remaining in the soluble fraction (Allen et al., 2004; Derkatch et al., 1996), was not inhibited by expression of Hsp104-A503V to such an extent as was OT56 (data not shown). One possibility is that toxicity of Hsp104-A503V is due to increased sequestration of the soluble Sup35 protein by prion aggregates. As Sup35 is an essential protein working in termination of translation, such sequestration may inhibit growth of the "strong" [PSI$^+$] variants where amount of the remaining soluble Sup35 is already low. To test this hypothesis, a high-copy plasmid expressing only the Sup35C domain was introduced into the [PSI$^+$] strain together with the Hsp104-A503V expression construct. Sup35C is completely functional in translation, but is not sequestered by the [PSI$^+$] prion aggregates, as it lacks the Sup35N prion domain. As expected, Sup35C counteracted toxicity of Hsp104-A503V in the [PSI$^+$] background (Figure 3.3 C).

Next we checked whether Hsp104-A503V influences the size of the Sup35 prion aggregates. These experiments employed the construct containing the Sup35N (prion) and M (middle) domains fused to GFP. Inclusion of this tagged protein into the prion aggregates makes them detectable by fluorescence microscopy (Chernoff et al., 2002). In the [PSI$^+$] cells, Sup35NM-GFP, expressed under the endogenous P_{SUP35} promoter, usually shows diffused fluorescence pattern (Bailleul et al., 2000; Wegrzyn et al., 2001), due to the fact that the Sup35^{PSI+} aggregates are usually so small in these conditions that they can hardly be recognized. However, in the presence of Hsp104-A503V, the

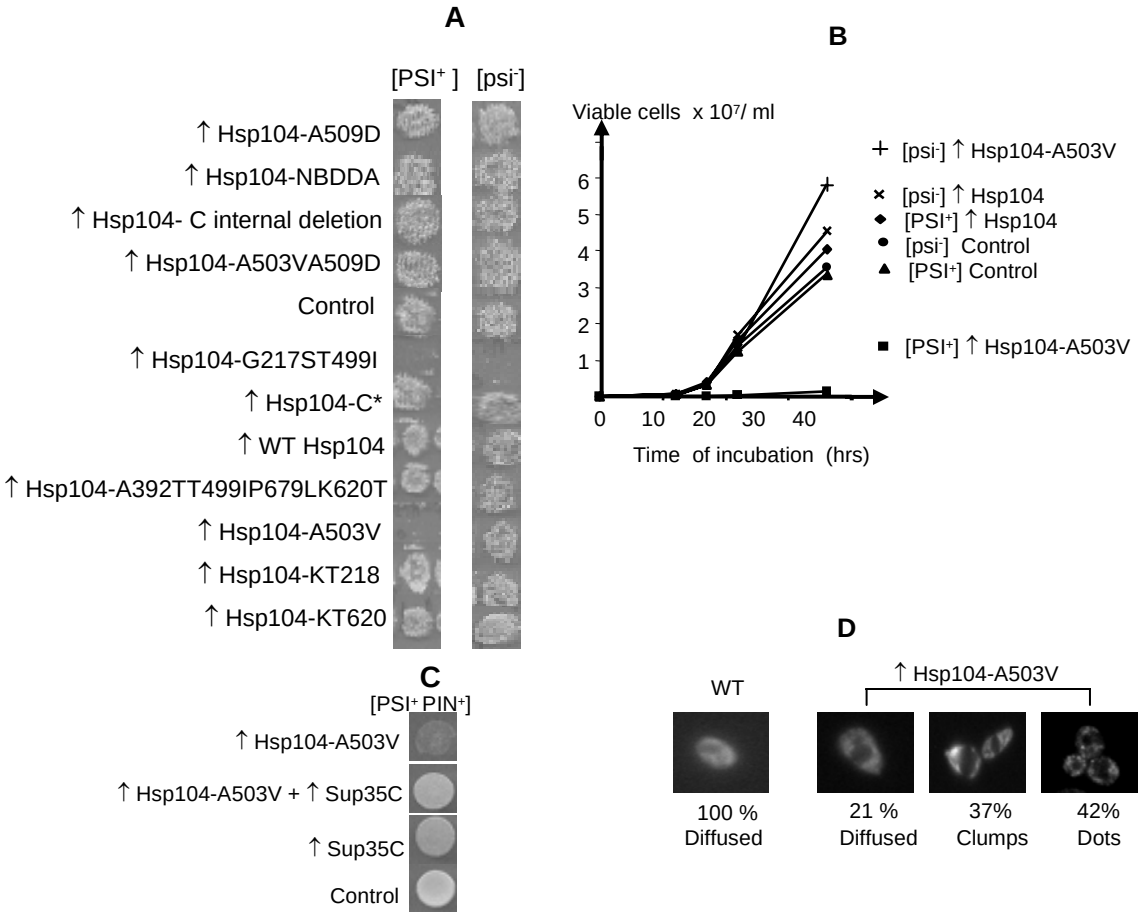


Figure 3.3 [PSI+] dependent toxicity of the Hsp104-A503V mutant derivative
A. Expression of Hsp104-A503V inhibits growth of the [PSI+] cells while expression of Hsp104-G217ST499I is toxic regardless of the prion background. Plates were photographed after 3 days of incubation (second passage) on the -His/Gal medium, used to induce the Hsp104 constructs. Data for [PSI+ PIN+] and [psi- PIN+](shown) and [PSI+ pin-] (not shown) was same as [PSI+ PIN+] . B. Quantitative assay demonstrating growth inhibition of [PSI+] cells expressing Hsp104-A503V. Cultures were grown in the liquid -His/Gal+Raf medium to maintain and induce the Hsp104-A503V and control constructs. Growth rate of the [PSI+] culture overexpressing Hsp104-A503V is statistically different from growth rate of all other cultures; differences between other cultures are statistically insignificant. C. Hsp104-A503V toxicity is ameliorated by overproducing Sup35C in [PSI+ PIN+] cells. Cultures contained the P_{GAL}-*HSP104-A503V* plasmid, high-copy *SUP35C* plasmid and empty controls in all possible combinations. Plates were photographed after 4 days of incubation (second passage) on the -Ura-His/Gal medium, which is selective for both plasmids and induces the Hsp104-A503V construct. D. Effect of Hsp104-A503V on the Sup35NM-GFP aggregation. Cultures were grown in the Gal+Raf medium, which is selective for both P_{GAL}-*HSP104-A503V* (or control) and P_{SUP35}-*SUP35NM-GFP* plasmids, and induces the P_{GAL}-*HSP104-A503V* construct. All cells expressing Sup35NM-GFP alone show diffused fluorescence, while cultures co-expressing Hsp104-A503V with Sup35NM-GFP contain cells with detectable clumps and dots. Numbers of cells are the averages of two counts, with at least 100 cells counted in each case.

same P_{SUP35}-*SUP35NM-GFP* construct produced large detectable clumps or dots (Figure 3.3 D). Thus, expression of Hsp104-A503V increased the size of Sup35 aggregates. Possibly, increased Sup35 aggregation contributes to cell toxicity. Such an effect of Hsp104-A503V would be similar to consequences of Sup35 (or Sup35N/NM) overproduction in the [PSI$^+$] background, which results in both appearance of large detectable Sup35 clumps (Patino et al., 1996) and cell toxicity (Chernoff et al., 1992;Ter-Avanesyan et al., 1993; Derkatch et al., 1996).

Effects of the yeast prion [PSI$^+$] and chaperone Hsp104 on Q103 toxicity

The poly-Q extended (Q103) exon 1 of human Htt fused to GFP is toxic when expressed under the galactose-inducible (P_{GAL}) promoter in the yeast strain containing endogenous QN-rich protein Rnq1 in the prion form, but not in the isogenic strain lacking a prion (Meriin et al., 2002). We have now demonstrated that [PSI$^+$], prion form of another protein with a QN-rich domain, release factor Sup35 (Chernoff, 2001; Chernoff et al., 2002), also promotes Q103 toxicity (Figure 3.4 A). This data was generously provided by G. Newnam (Chernoff Lab). Effects of [PIN$^+$] and [PSI$^+$] on Q103 toxicity appear to be additive (data not shown).

Overproduction of the yeast chaperone Hsp104 is known to eliminate [PSI$^+$] (Chernoff et al., 1995) but not [PIN$^+$] (Derkatch et al., 1997). In agreement with these data, we have shown that overproduction of wild-type Hsp104 counteracts Q103 toxicity only in a [PSI$^+$] background and not so when [PSI$^+$] is absent (Figure 3.4 B).

Mutant alleles of Hsp104 counteract Q103 toxicity

The double K-to-T substitution at aa positions 218 and 620 of Hsp104 (Hsp104-KT) inactivates both nucleotide binding domains of Hsp104 (NBD1 and NBD2) and disrupts the function of wild-type Hsp104 in a dominant negative fashion (Chernoff et al.,

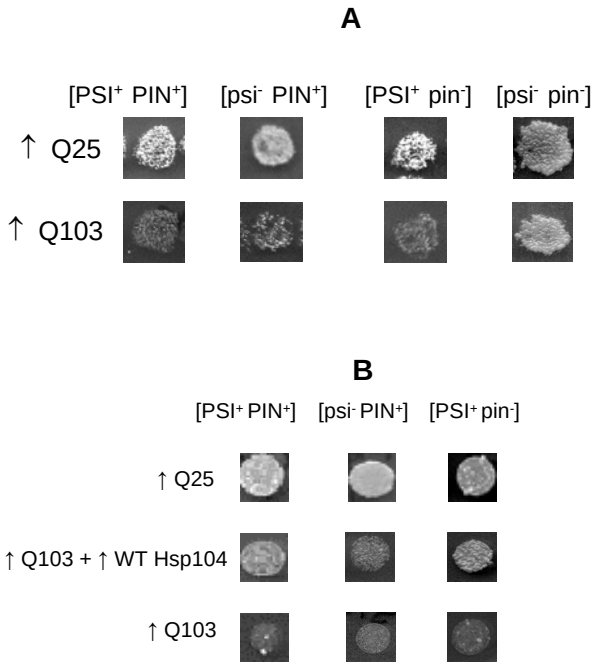


Figure 3.4 Effects of yeast prions and Hsp104 on Q103 toxicity
A. Presence of either [PSI+] or [PIN+] prion is required and sufficient for Q103 toxicity. Medium is selective for plasmid (-Ura) and contains galactose to induce the poly-Q constructs. Plates were photographed after 3 days of incubation (second passage). B. Excess Hsp104 ameliorates poly-Q toxicity only in [PSI+] cells but not in [psi- PIN+] cells. Strains contained the *HIS3* marked Hsp104 overexpressing plasmid (WT Hsp104) or empty control, in addition to a poly-Q construct. Plates were photographed after 7 days of incubation (second passage) on the -Ura-His/Gal medium, which is selective for plasmids and induces poly-Q and Hsp104 constructs. In both A and B, no differences in growth were detected on the glucose (Glu) medium (data not shown) and at least 8 individual transformants tested in each case with typical representatives shown.

1995; Schirmer et al, 1998). In contrast to overproduced wild-type Hsp104, mutant Hsp104-KT cures yeast cells not only of [PSI^+] (Chernoff et al., 1995) but also of [PIN^+] (Wegrzyn et al., 2001). As expected, expression of Hsp104-KT counteracts Q103 toxicity not only in the [PSI^+] strains but also in the [psi^- PIN^+] strains, apparently by curing cells of prions (data not shown), thus reproducing an effect of the *hsp104* deletion (Meriin et al., 2002). Single dominant negative NBD mutants KT218 and KT620 also exhibit anti-toxicity (data not shown).

Surprisingly, we have isolated a presumably wild-type *HSP104* clone from a widely used cDNA yeast library, that counteracted Q103 toxicity in both [PSI^+] (not shown) and [psi^- PIN^+] (Figure 3.5 A) backgrounds. DNA sequencing revealed that this clone of *HSP104* actually contained a G-to-C substitution at the nucleotide position 906, resulting in K-to-N substitution at the aa position 302 within NBD1. It was found to inhibit the thermotolerance function of Hsp104 in a dominant-negative fashion like Hsp104-KT (Figure 3.5 B) and cure endogenous prions (Table 3.2).

Next, we checked whether *HSP104* mutant alleles obtained from S. Lindquist and checked previously for endogenous prions afffect poly-Q toxicity. Indeed, mutant alleles of *HSP104* with substitutions in the middle region and C-terminal region, counteracted Q103 toxicity with varying degrees depending on the prion background (Figure 3.6 A). While expression of Hsp104-G217ST499I is toxic regardless of the prion background, this mutant could not be studied for poly-Q toxicity (Figure 3.3 A and table 3.2). Mutant alleles of Hsp104 like *HSP104-A503V* and *HSP104-C** counteracted Q103 toxicity in the [psi^- PIN^+] strain (figure 3.6 A, B) without curing yeast cells of prions in the absence (Table 3.2) and presence of poly-Q (Table 3.3). Hsp104-C* could counteract Q103 toxicity in [PSI^+] background too (Figure 3.6 A).

As A503V mutation counteracted poly-Q toxicity without curing [PIN^+], we have checked its effects along with the C* mutation on Q103 aggregation in the [psi^- PIN^+]

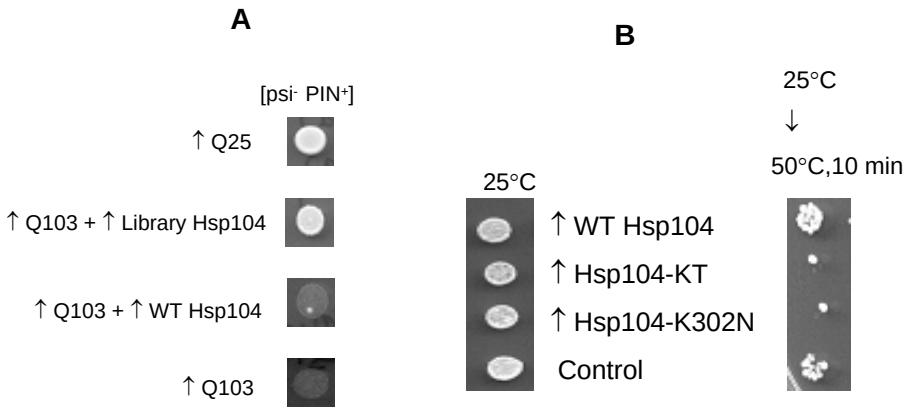


Figure 3.5 Effects on the *HSP104* clone isolated from the yeast cDNA library on A. Q103 toxicity and B. thermotolerance of WT Hsp104
A. Strains contained the *URA3*-based Hsp104 plasmid (as shown) or empty control, in addition to the *TRP1* poly-Q constructs. Plates were recorded after 3 days of incubation (second passage) on the -Ura-Trp/Gal medium, which is selective for plasmids and induces both Hsp104 and poly-Q constructs. The *HSP104* clone isolated from the yeast cDNA library (Library Hsp104) reduces Q103 toxicity in the [psi$^-$ PIN$^+$] background, in contrast to wild-type Hsp104.
B. [psi$^-$ pin$^-$] strain GT17 was transformed with the *URA3*-based WT Hsp104, Hsp104K302N, Hsp104-KT or empty control. 10 fold serial dilutions of the heat-shocked samples (50°C for 10 mins) and control samples on –Ura medium is shown. Samples before heat-shock are also shown (see materials and methods for more details on the procedure). Hsp104K302N like dominant negative Hsp104-KT mutant affects the thermotolerance function of WT Hsp104.

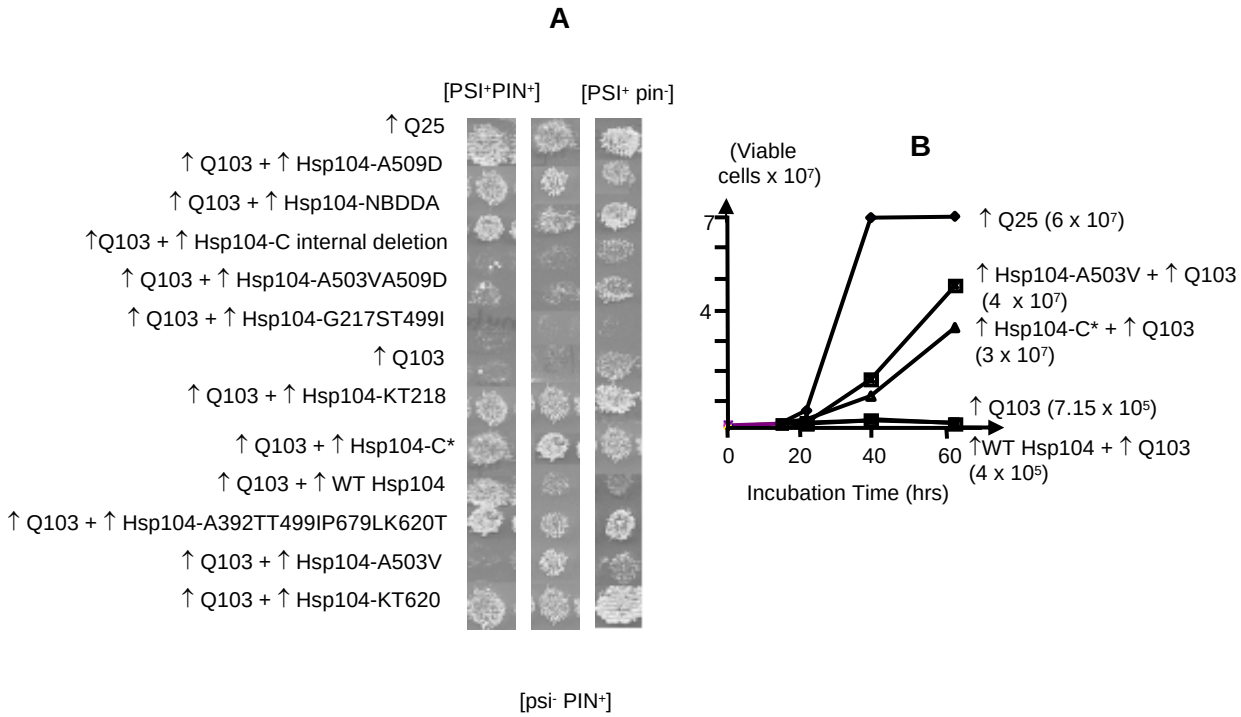


Figure 3.6 Effect of mutant derivatives of Hsp104 (Lindquist Lab) on Q103 toxicity

A. Antitoxic effect of the mutant alleles of Hsp104 vary depending on the prion background. Strains contained the *HIS3*-based Hsp104 plasmid (as shown) or empty control, in addition to the *URA3* poly-Q constructs. Plates were photographed after 7 days of incubation (second passage) on the -Ura-His/Gal medium, which is selective for the plasmids and induces both Hsp104 and poly-Q constructs. Amongst all the *HSP104* alleles tested,only *HSP104-A503V* and *HSP104-C** antagonized Q103 toxicity without the curing of prions [PSI$^+$] and [PIN$^+$] (seeTables 3.2 and 3.3) B. Quantitative assay confirming the antitoxic effect of the *HSP104-A503V* and *HSP104-C** in [psi$^-$ PIN$^+$] strain OT60: Cultures were grown in the liquid –Ura-His medium containing galactose and raffinose (Gal+Raf) to induce poly-Q and Hsp104 constructs. (see materials and method for procedural details and plasmids used)

Table 3.3 Effect of Hsps on yeast prions in the presence of poly-Q

Overexpressor combinations	Tested for [PSI$^+$]			Tested for [PIN$^+$]		
	[PSI$^+$]	[psi$^-$]	Total	[PIN$^+$]	[pin$^-$]	Total
↑ Q103	48	0	48	48	0	48
↑ WT Hsp104 + ↑ Q103	0	48	48	48	0	48
↑ Hsp104-A509D + ↑ Q103	21	27	48	18	30	48
↑ Hsp104-A503V + ↑ Q103	Not applicable*			48	0	48
↑ Hsp104-A503VA509D + ↑ Q103	3	45	48	40	8	48

* Hsp104-A503V is toxic to [PSI$^+$]

background. Normally, Q103 forms large “clumps” in about 40% of the [psi$^-$ PIN$^+$] cells, and either “dots” or both small clumps and dots in the rest of them (Figure 3.7 A, B). In contrast, cells co-expressing either Hsp104-A503V or Hsp104-C* with Q103 showed essentially no large clumps, instead most of them contained dots (Figure 3.7 B). This shows that expression of Hsp104-A503V and Hsp104-C* decreases size of Q103 aggregates, suggesting a possible mechanism for anti-toxicity.

Prion-dependent Q103 aggregation in yeast is associated with a defect of endocytosis, that is one of the likely causes for Q103 toxicity (Meriin et al., 2003). We have monitored the effect of Q103 aggregation on endocytosis by using the lipophilic fluorescent dye FM4-64 (see materials and methods for more details). This dye binds to the cell membrane and is internalized through the endocytic pathway. In cells with normal endocytosis, FM4-64 forms a distinct ring around the vacuole at 45 min after dye addition, reflecting the fusion of dye-stained endosomes with the vacuolar membrane. In contrast, cells with large Q103 clumps, and about 50% of cells with both small clumps and dots contained no such fluorescent rings, indicating a defect in endocytosis. Cells

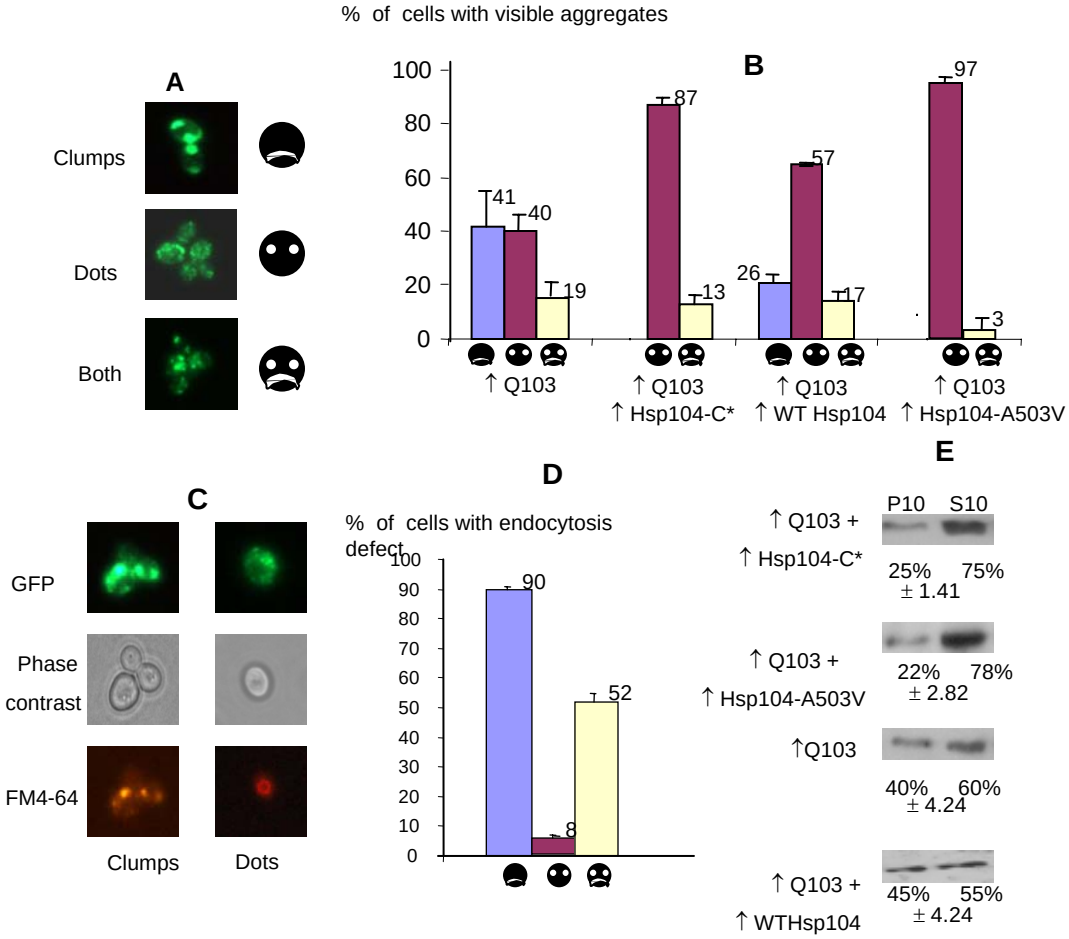


Figure 3.7 Effects of the Hsp104-A503V and Hsp104-C* mutant derivatives on Q103 aggregation and endocytosis.

A. Types of aggregates observed in Q103 overexpressing cells. Cultures were grown in the liquid Gal+Raf medium to induce the poly-Q construct. Three distinct classes of cells with aggregates were observed: - cells containing large clumps; cells containing dots; - cells containing both clumps and dots. B. Expression of Hsp104-A503V and Hsp104-C* decreases proportions of cells with clumps and increases proportion of cells with dots. Average number from 2 repeats for each of 2 independent cultures is given in each case. Error bars indicate standard deviations. C and D. Clumps but not dots block endocytosis. Ring-like staining of the vacuole with FM4-64 is a marker of normal endocytosis. Majority of cells with dots show rings, while majority of cells with clumps and 50% of cells with both clumps and dots do not, indicating a defect in endocytosis. Note that partial leakage of GFP fluorescence through red filter occurs in the cells with large clumps. Data were homogenous for all plasmid combinations. Error bars indicate standard deviations.E. Expression of Hsp104-A503V and Hsp104-C* increases proportion of soluble Q103 protein.Proteins were isolated after 24 hrs of growth in synthetic Gal+Raf medium selective for plasmids, and fractionated as described in materials and methods. No differences in the proportion of Q103 precipitated at 800 g were seen (not shown). Proportions of Q103-GFP protein in pellet (P) and supernatant (S) fractions obtained at 10,000 g were determined by densitometry. Standard deviations are shown.

with dots usually exhibited FM4-64 rings confirming that in the absence of large aggregates, endocytosis was not affected (Figure 3.7 C, D). As expression of Hsp104-A503V and Hsp104-C* increased proportion of cells with dots and almost eliminated large clumps, defect of endocytosis was essentially not seen in these cultures. Differential centrifugation assay confirmed that co-expression of Hsp104-A503V/Hsp104-C* with Q103 shifted a portion of the Q103 protein from the fraction pelletable at 10,000 g to the supernatant fraction (Figure 3.7 E). At higher centrifugation speed (200,000 g) essentially all Q103 was precipitated independently of the presence or absence of the mutant Hsp104 proteins, while non-expanded Q25 control remained soluble (data not shown). These results are in agreement with the fluorescence microscopy data and show that expression of Hsp104 mutants does not solubilize Q103 aggregates but leads to a decrease in aggregate size, so that higher speed is needed for the efficient precipitation of aggregates. As total levels of Q103 were not affected by mutants (data not shown), decrease in aggregate size should be accompanied by an increase in the number of aggregated units. Taken together, our data confirm that Hsp104-A503V and Hsp104-C* ameliorates Q103 toxicity by counteracting formation of the large aggregates, which inhibit endocytosis.

Amongst the middle domain mutant alleles of Hsp104 that carried triple aminoacid substitutions and were checked previously for effects on yeast prions- *HSP104-L445R,A448Asp,L455K* (MII) counteracts poly-Q associated toxicity in strains irrespective of the presence or absence of either prions [PSI^+] (Figure 3.8 A) or [PIN^+] (data not shown). MII thus showed poly-Q anti-toxicity because of loss of endogenous prions [PIN^+] and [PSI^+] (Table 3.2). Strains expressing *HSP104-A479Asp,L483K,L486R* did not grow regardless of the prion background (Figure 3.8 A) as seen before (Table 3.2) and therefore its effects could not be tested for anti-toxicity.

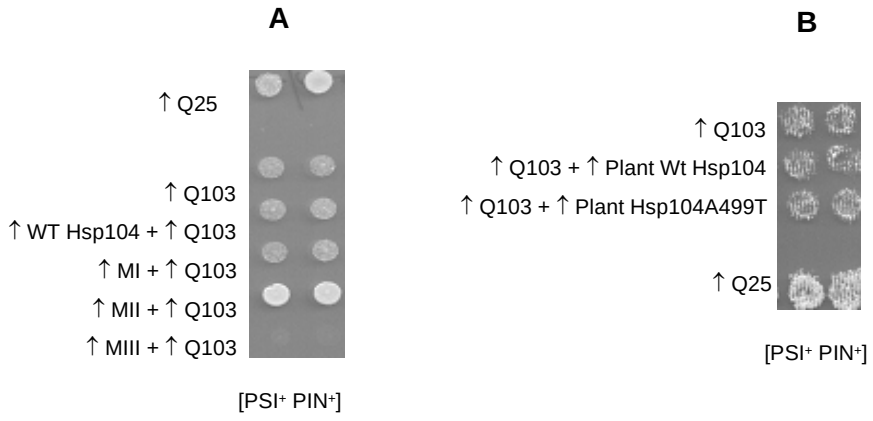


Figure 3.8 Effect of middle domain mutants of yeast and plant Hsp104 on Q103 toxicity

A. MIII is toxic while MII is able to counteract poly-Q toxicity regardless of the prion background. Yeast strains (mentioned below) were transformed with plasmids “Q25”- pYES-Q25trp + pRS316GAL, “Q103”- pYES-Q103trp + pRS316GAL, “WT Hsp104 +Q103 “- pYSGAL104 + pYES-Q103trp, “MI +Q103” – pYSGAL104-MI + pYES-Q103trp, “MII + Q103” - pYSGAL104-MII + pYES-Q103trp, “MIII +Q103”- pYSGAL104-MIII + pYES-Q103trp. Transformants were grown on –Ura-Trp and spotted onto –Ura-Trp/Gal to induce the constructs.Plate is photographed after 5 days of incubation. See materials and methods for details on mutant plasmids. B. Both plant Hsp104-wild-type and middle domain mutant cannot counteract poly-Q toxicity regardless of the prion background. Yeast strains (mentioned below) were transformed with plasmids “Q103”- pYES2-103Q + pLA1 , “Q103 + Plant Wt Hsp104”-pYES2-103Q + pLA1- plantHsp104Wt , “Q103 + Plant Hsp104A499T”-pYES2-103Q + pLA1- plantHsp104MT, “Q25” – pYES2-Q25 + pLA1. Transformants were grown on –Ura-His and velveteened onto –Ura-His/Gal + Raf. Plates were photographed after 5days of incubation. For both A and B, typical representatives of [PSI^+ PIN^+] are shown. [psi^- PIN^+]- OT60 and [PSI^+ pin^-] –GT490 data not shown was similar.

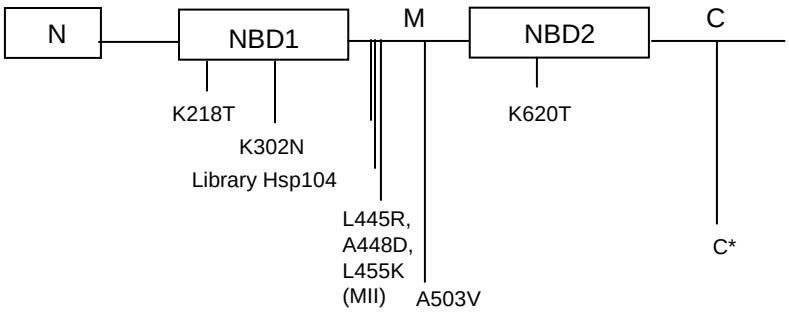


Figure 3.9 Domain architecture of the Hsp104 protein showing locations of mutations exhibiting an anti poly-Q toxicity effect
NBD1 and NBD2 - nucleotide binding domains 1 and 2, respectively; N, M and C – the N-terminal, middle and C-terminal regions of Hsp104, respectively.

Both Plant Wild-type and mutant Hsp104 containing G to A substitution at position 1495-corrosponding to A499T did not antagonize poly-Q toxicity when tested in [PSI^+ PIN^+] (Figure 3.8 B), [psi^- PIN^+] and [PSI^+ pin^-] strains (data not shown). Here, irrespective of no loss of endogenous yeast prions [PSI^+] and [PIN^+] (Table 3.2), yeast Hsp104 –both wild-type and mutant were not able to overcome Q103 toxicity. Location of mutations in Hsp104 showing anti poly-Q toxicity is shown in figure 3.9.

Discussion

Comparison between prion-curing effects of Hsp104 inactivation

[PSI^+] loss by GuHCl follows a slow generation dependent kinetics with a long lag period required to decrease the average copy number of prion aggregates. Based on the calculation of kinetics of GuHCl–induced loss, [PSI^+] strain used in that work contained about 60 proliferating $Sup35^{PSI+}$ units per cell (Eaglestone et al., 2000) . Kinetics of [PSI^+] curing in our strains (see Figure 3.1) was generally similar to those reported previously (Eaglestone et al., 2000), although weak [PSI^+] was lost faster than strong [PSI^+] as expected (see Figure 3.1). However, [PSI^+] loss induced by Hsp104-KT did not show a significant lag period and followed rapid kinetics, strikingly different from that of GuHCl-induced loss in the same conditions (Figure 3.1). These differences contradict the previous model suggesting that GuHCl cures yeast cells of [PSI^+] (and possibly other prions) by inactivating Hsp104 (Eaglestone et al., 2000; Glover and Lindquist, 1998). [PSI^+] loss cannot be therefore explained to occur by simple dilution in cell divisions. Alternative explanation could be that, in addition to the proliferation defect, Hsp104 inactivation affects the state of preexisting prion units or that GuHCl mediated inactivation of Hsp104 is incomplete and is sufficient only for blocking [PSI^+] proliferation but not for altering the parameters of preexisting $Sup35^{PSI+}$ aggregates.Moreover, [PIN^+]

loss after Hsp104 inactivation was even more rapid than [PSI$^+$] loss (Figure 3.1 D and Table 3.1).

Interaction of Hsp104 and Hsp70-Ssa in prion maintenance

Hsp70-Ssa1 protects [PSI$^+$] from curing by excess Hsp104 (Newnam et al., 1999). Excess Ssa1 also increases nonsense suppression by [PSI$^+$] (Newnam et al., 1999) while mutation in SSA1 decreases [PSI$^+$] stability and eliminates [PSI$^+$] in combination with deletion of another member of the Hsp70 family-SSA2 (Jung et al., 2000). The data indicates that Ssa1 favors [PSI$^+$] propagation. However, excess Ssa1 failed to protect [PSI$^+$] by curing with hsp104 deletion (Newnam et al., 1999) or by Hsp104-KT (Figure 3.2 B). Moreover the anti-[PSI$^+$] effect of Hsp104-KT was enhanced by excess Ssa1 (Figure 3.2 C). Based on the model proposed for Hsp104-Hsp70Ssa concerted action, inactivation of Hsp104 leads to slowing of the disaggregation process leading to monomerization. In contrast increased levels of Ssa result in intensified assembly of new aggregates. This could result in accumulation of large aggregates losing prion activity.

Role of endogenous prions in poly-Q aggregation and toxicity

Our data confirms previous observations showing that the presence of endogenous yeast QN-rich proteins in a prion form promotes poly-Q aggregation (Meriin et al., 2002; Osherovich and Weissman 2001) and toxicity (Meriin et al., 2002), and demonstrate that [PSI$^+$], a prion form of Sup35, is promoting toxicity of the Htt-derived poly-Q construct in the same way as does [PIN$^+$], a prion form of Rnq1 (Figure 3.4 A). These results, implicating endogenous QN-rich prions as susceptibility factors in the

poly-Q disorders, could be relevant to mammalian cells, containing a lot of proteins with potentially prionogenic QN-rich domains (see Michelitsch and Weissman, 2000).

Prion domains of Sup35, and possibly of Rnq1 are composed of “aggregation modules”, or QN-rich stretches, and “propagation modules” - in case of Sup35, oligopeptide repeats (Chernoff, 2004; Osherovich et al, 2004). QN-rich stretches form stable intermolecular β-sheets, leading to generation of aggregates with fibrillar morphology (Perutz, 1999), characteristic for both prions and poly-Q proteins such as Htt. It is possible that QN-rich prion aggregates serve as aggregation “seeds” for the poly-Q derivatives of Htt (Meriin et al., 2002). “Propagation modules” are reguired for propagation of aggregates in the cell divisions via repetitive cycles of the chaperone-mediated aggregate “shearing”, generating new “seeds” (Chernoff, 2004; Osherovich et al, 2004). Poly-Q fragments of Htt apparently lack propagation capabilities on their own (Osherovich et al, 2004), so that in the absence of endogenous prions, poly-Q aggregates are not only rarely formed but also incapable to persist in the yeast cells. Possibly, association with prions confines a self-perpetuating capability to the whole prion/poly-Q complex. Direct biochemical assays confirm that aggregated Q103 is associated with Rnq1 in the yeast cells (Meriin et al., 2003).

Role of the chaperone Hsp104 in poly-Q aggregation and toxicity

Numerous previous studies have shown that the presence of Hsp104 is required for poly-Q aggregation in yeast (Krobitsch and Lindquist,2000; Cao et al., 2001; Kimura et al., 2001; Meriin et al.,2002). Hsp104 overproduction reduced poly-Q aggregation at least in some yeast strains (Krobitsch and Lindquist, 2000) and in the heterological model of *C. elegans* (Satyal et al.,2000). However, Hsp104 is required for propagation of all known endogenous yeast prions, including [PSI^+] (Chernoff et al., 1995) and [PIN^+] (Derkatch et al., 1997). Apparently, it serves as a major factor promoting aggregate

"shearing" (Chernoff, 2004). Therefore, previously reported effects of Hsp104 on poly-Q aggregates could be mediated by its effects on the endogenous prions.

Indeed, the dominant negative Hsp104 derivatives with mutations in NBDs (both the previously described Hsp104-KT218, 620 and newly found Hsp104-K302N) or in the middle region of Hsp104 (Hsp104-A509D and Hsp104-L445RA448DL455K-MII) inhibited both poly-Q toxicity and Hsp104 function in prion maintenance (Figure 3.5 A, 3.6 A, 3.8 A and Table 3.2). Loss of poly-Q toxicity was accompanied by a loss of the endogenous prion(s), implicating prion elimination as a major cause of the toxicity relief. In contrast, overproduction of Hsp104, which is known (Chernoff et al., 1995, Derkatch et al., 1997) and confirmed to solubilize and eliminate [PSI^+] but not [PIN^+], reduced poly-Q toxicity only in the [PSI^+] strains but not in the [psi^- PIN^+] strains (Figure 3.4 B), confirming that excess Hsp104 influenced poly-Q via prion elimination.

However, in the case of Hsp104-A503V and Hsp104-C* mutants, toxicity relief (Figure 3.6 A, B) was not accompanied by elimination of a prion (Tables 3.2 and 3.3) or loss of a thermotolerance function of Hsp104 (Schirmer et al., 2004). Anti-toxicity effect by these mutants was apparently due to decreased size of poly-Q aggregates (Figure 3.7 B): expression of mutant Hsp104 reduced proportion of the cells with large poly-Q clumps (Figure 3.7 B), which exhibit a defect in endocytosis (Figure 3.7 C, D), a landmark of prion-dependent poly-Q toxicity in yeast (Meriin et al., 2003). Thus, our data provide the first evidence that at least some mutant derivatives of Hsp104 are capable of modulating poly-Q aggregation without eliminating yeast prions.

Differential effects of Hsp104-A503V on poly-Q and [PSI^+] aggregates

Quite remarkably, expression of the mutant Hsp104-A503V protein was incompatible with the presence of the strong variant of [PSI^+] prion (Figure 3.3 A, B). This effect was counteracted by simultaneous expression of the Sup35C region,

incapable of aggregation due to lack of prion domain (Figure 3.3 C). Thus, incompatibility between Hsp104-A503V and [PSI$^+$] was most likely due to shortage of functional Sup35.

Indeed, fluorescence microscopy confirmed that size of the Sup35 aggregates is increased in the [PSI$^+$] strain expressing Hsp104-A503V (Figure 3.3 D). This could occur due to either promotion of aggregation or defect in aggregate "shearing". For instance, underexpression of Hsp104 or expression of the dominant negative Hsp104 allele (Hsp104-KT218,620) in the [PSI$^+$] strains also resulted in increased size of the Sup35 clumps, apparently due to defect in "shearing" (Wegrzyn et al., 2001). However, cell growth was not inhibited in these cases, probably due to the fact that shearing-defective Sup35 aggregates were quickly lost from the population. This apparently does not occur in case of the Hsp104-A503V mutant, indicating that this mutation probably makes Hsp104 capable of promoting aggregate growth by facilitating prion conversion rather than by inhibiting aggregate shearing. Indeed, increased rate of prion conversion would facilitate sequestration of the newly synthesized Sup35 by prion aggregates and in this way may inhibit growth. Such an explanation also agrees with data showing that Hsp104 promotes *in vitro* Sup35 aggregation in certain assays (Shorter and Lindquist, 2004). Observations that Hsp104-A503V remains functional in thermotolerance (Schirmer et al., 2004) and does not antagonize [PIN$^+$] (Table 3.2 and Table 3.3) also support gain-of-function (rather than impairment of function) model of its action.

Schirmer et al. have previously reported that the Hsp104-A503V and double Hsp104-A503V, A509D mutants inhibit growth of some yeast strains at 37^{o}C. The prion status of the strains used in that paper was not reported, but at least in the case of double mutant, growth inhibition probably did not depend on [PSI$^+$], as it was not counteracted by Sup35C, in contrast to our data. Our [psi$^-$] strain, coming from a

different genotypic background, did not show temperature sensitivity with Hsp104-A503V.

Possible molecular mechanisms of the Hsp104-A503V effects

According to the previous data of S. Lindquist lab (Cashikar et al., 2002), the A503V mutant exhibits increased background ATPase activity but can not further increase ATP hydrolysis of NBD1 in response to substrate binding at the C-terminus. We propose that these alterations of the Hsp104 function have different consequences, depending on the type of aggregates Hsp104 is dealing with. In case of non-perpetuating poly-Q aggregates, increased background ATPase activity may lead to increased aggregate shearing, and therefore, to decreased aggregate size. Hsp104 interaction with the self-perpetuating (prion) aggregates of Sup35 is more complex. One possibility is that inability of the mutant Hsp104 to undergo fine tuning of the NBD results in shifting the balance between its “shearing” and “conversion-promoting” activities, that facilitates aggregate growth and sequestration of the newly synthesized Sup35 protein by aggregates.

CHAPTER 4

EFFECTS OF CHAPERONES ON Q-RICH PROTEIN AGGREGATES-PART II

Background

Hsp70

The Hsp70 group of chaperones have been implicated in numerous cellular processes some relevant to this study are regulation of heat shock response, protein translocation, cytosolic protein folding (for review see Craig et al., 1999) and translation (Horton et al., 2001). The Ssa subfamily is one of the major heat shock inducible cytosolic chaperones in yeast and consists of four proteins (Ssa1, Ssa2, Ssa3 and Ssa4). Ssa1 and Ssa2 share 97% identity, and the more distantly related Ssa3 and Ssa4 share 80% identity to each other and Ssa1 (Craig et al., 1985). Ssa2 is constitutively expressed at high levels during steady state growth. Ssa1 is present at a lower level during steady state growth, but is highly induced during heat-shock along with Ssa3 and Ssa4. Members of the Hsp70-Ssa family together with Hsp104 provide thermotolerance in vivo (Sanchez et al., 1993) and in protein disaggregation in vitro (Glover and Lindquist, 1998). Along with the known effects of Hsp70 on yeast prions, Hsp70 was shown in vitro to interact with fragments of Htt containing the poly-Q tract and inhibit the ordered assembly of SDS-resistant HD exon-1 fibrils. The Hsp70/Hsp40 system was also shown to mitigate the neurotoxicity of polyQ expanded proteins in a Drosophila disease model (Warrick et al., 1999; Kazemi-Esfarjani and Benzer, 2000). Inhibition of poly-Q aggregation *in vivo* was in some cases associated with anti-toxicity.

Members of the Hsp70-Ssb proteins (Ssb1 and Ssb2) make up the second subfamily of cytosolic Hsp70s that share 99% sequence identity. Ssbs are non-essential

proteins that are constitutively expressed during steady-state growth. Ssbs share 60% aminoacid identity to the Hsp70-Ssa subfamily (Craig and Jacobsen, 1985). Ssbs of *Saccharomyces cerevisiae* are ribosome-associated molecular chaperones, which can be cross-linked to nascent polypeptide chains. Close association of Ssb with translating ribosomes makes the strain carrying deletions of *SSB1* and *SSB2* sensitive to translation inhibiting drugs like aminoglycoside, paromomycin (Par^s) and Hygromycin (Hyg^S) (Nelson et al., 1992). In addition they also are cold sensitive and osmosensitive. *ssb1,2*Δ also produces sensitivity to GuHCl (Ghc^s) (Chernoff *et al.,* 1999). In contrast to Ssa1, the Ssbs consistently act as [PSI^+] antagonist, while with Htt-Q103, there hasn't been any reporting of Ssb effects.

Hsp70 has two major functional domains: an ATPase domain and a substrate binding domain. Ssb in particular is structurally composed of three domains: highly conserved 44-KDa ATPase domain (amino acids 1-400), followed by a polypeptide-binding domain that is less conserved (amino acids 401-507) and a variable extreme C-terminal domain (amino acids 507-613). An intact peptide-binding domain is essential and an alteration of a conserved residue in the peptide-binding cleft (V442) affects its function (Pfund et al., 2001).

Hsp40

Hsp40 regulate Hsp70 ATPase activity (Cyr and Douglas, 1994) and can also present unfolded peptides to Hsp70 (Qian et al., 2002). Members of the Hsp40 family have been shown to regulate prions.Like Hsp70, Hsp40 can also modulate other abnormal non-prion Q-rich protein aggregates like Htt.

In this chapter, we have systematically examined the effects of various yeast chaperones of the Hsp70/Hsp40 complex along with other yeast chaperones Hsp82 (Hsp90 family) and small yeast chaperoneHsp26 on poly-Q aggregation and toxicity in

the series of isogenic yeast strains that differ only by prion composition. We also study the effects of Hsp70-Ssa/Ssb on [PSI$^+$].

Materials and methods

Yeast strains

Yeast strains unique to this study are described here. Other commonly used strains are described in Chapter 2 and Table 2.1. GT146 is [PSI$^+$ PIN$^+$] *ssb1,2* Δ , GT157 is [psi$^-$ PIN$^+$] *ssb1,2* Δ , GT202 and GT203 are [PSI$^+$] derivatives of GT157 obtained by overexpression of Sup35N. They exhibit decreased non-sense suppression in the presence of wild-type Ssb1 protein (Chernoff et al., 1999). GT310 is the "Sup35PS-only" [PSI$^+$]^{PS} strain as described in Chernoff et al., 2000.The *Pichia-Saccharomyces* prion is designated as [PSI$^+$]^{PS} and Sup35PS indicates that the prion forming domain of Picha Sup35 is fused to the MC domain of *Saccharomyces* Sup35. The strain PJ69-4A alias OT59 (James et al., 1996) was used for yeast two-hybrid assays.

Plasmid constructs

Some plasmids specific to this study are described here, for all other commonly used plasmids refer to Chapter2. Sequence analysis of the plasmid pRDW30 carrying P_{SSA1}-*SSA1-21* (Jung et al., 2000) revealed a base change resulting in a substitution of tryptophan for leucine at amino acid residue 483 (L483W).

pACT-SSB1 was constructed by ligating the BamH1 XhoI fragment containing *SSB1* from pTEF-SSB1 to pACT cut with the same enzymes. pACT-SSA1 was constructed by ligating the BamH1 fragment of pTEF-SSA1 to pACT cut with BamH1. This construction was done with the help of an undergraduate student Heather Vanpelt (Chernoff Lab).

Medium used for Ssb studies

Complete medium YPD with 0.2mg/ml paromomycin, YPD + 35μgm/ml Hygromycin and YPD + 5mM GuHCL were used to monitor drug sensitivity of *SSB* mutants. Similarly, synthetic selective media –Ura + above mentioned addition of drugs was used to monitor sensitivity.

Two-hybrid assay

In vivo reconstitution of the split Gal4 activator as a result of physical interaction between proteins fused to different Gal4 domains was detected by growth on –Ade medium due to activation of the *GAL2::ADE2* reporter construct in the strain PJ69-4A as described previously (Bailleul et al.,1999 and references therein). The Ade^+ transformants in which *GAL2::ADE2* was activated presumably by physical interaction between two proteins (Ssa1 and Sup35Sc- from this study) were screened for activation of *GAL1::HIS3* by growing the transformants on –trp-leu-his medium in the presence of 1mM aminotriazole (AT) to eliminate the possibility of mutations that could activate transcription of the *ADE2* gene in a Gal4-independent fashion.

Results

The data on Hsp70 and Hsp40 effects on poly-Q toxicity is submitted for publication to the Journal of Biological Chemistry as a part of Gokhale et al., 2005 manuscript.

Effects of yeast Hsp70 family and other small chaperones Hsp82 and Hsp26 on Q103 toxicity

Yeast Hsp70 proteins of the Ssa subfamily aid in propagation of the yeast prion [PSI^+] (Newnam et al., 1999; Allen et al., 2003; Jung et al., 2000), and exhibit differential effects on the other yeast prion, [URE3] (Schwimmer and Masison, 2002; Roberts et al., 2004). The amino acid substitution of tryptophan for leucine at residue 483 (L483W) of the Ssa1 protein (also referred as Ssa1-21) results in reduced [PSI^+] stability characterized by decreased nonsense suppression, reduced prion 'seed' number, high mitotic loss of [PSI^+]. When Ssa1-21 is the only source of Ssa in cell ie in *ssa1Δssa2Δ* background, cells show [PSI^+] no more phenotype (Jung et al., 2000). Proteins of the other cytosolic Hsp70 subfamily, Ssb, consistently antagonize [PSI^+] in various assays (Allen et al., 2004;Chernoff et al., 1999; Chacinska et al., 2001). In contrast, Hsp26 and Hsp90 chaperones did not show any detectable effects on [PSI^+] (Newnam et al., 1999).

We have checked the effects of the individually overproduced Hsps on the prion-dependent Q103 toxicity in yeast. Among all yeast Hsp70 proteins tested (Ssa1, 2, 3 and 4, Ssa1-21 and Ssb1) (Figure 4.1 A, B, C), only overproduction of Ssa4 counteracted Q103 toxicity in the [PSI^+ PIN^+] strain (Figure 4.1 B), although it exhibited no detectable anti-toxicity in the [psi^- PIN^+] or [PSI^+ pin^-] backgrounds, and did not cure [PSI^+] at a significant level (Allen et al, 2004 and data not shown). Overproduction of Hsp26 or Hsp90 (Hsp82) did not exhibit any antitoxic effect on Q103 independently of which prions were present (Figure 4.1 D).

Differential effects of the Hsp40 chaperones on Q103 toxicity

Hsp40 and Hsp70 chaperones cooperate with Hsp104 in solubilization and refolding of heat-damaged proteins (Glover and Lindquist, 1998). One of the major yeast Hsp40 chaperones, Sis1, is also involved in [PIN^+] propagation (Sondheimer and

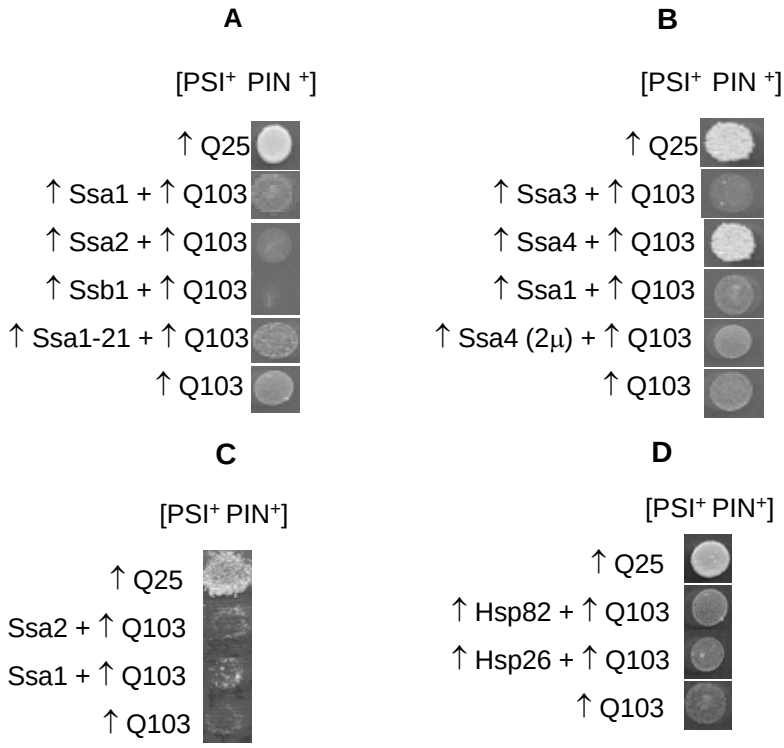


Figure 4.1 Effect of Hsp70, Hsp82 and Hsp26 chaperones on Q103 toxicity
A. *pGAL1-SSA1*, *pGAL1-SSA2*, *pGAL1-SSB1*, *pSSA1-SSA1-21* none exhibit anti-poly-Q toxicity effect. Medium used is selective for the plasmids -Ura-Trp/Gal + Raf and contains galactose to induce the poly-Q and chaperones that are under gal promoter. Plates were photographed after 10 days of incubation (second passage). Result for OT56 is shown. Data with OT60 and GT490 was similar and is not shown. Ssa1 expressed under *TEF* promoter also did not show any anti poly-Q toxicity effects (data not shown). B. Excess Ssa4 counteracts poly-Q toxicity only in [PSI$^+$ PIN $^+$] strain. Medium used was selective for plasmids and contained galactose to induce poly-Q and chaperones under gal promoter (-Ura-Leu/Gal + Raf). Plates were photographed after 7 days of incubation. *pGAL1-SSA3*, *pGAL1-SSA4*, *pSSA1-SSA1* and *pGAL1-SSA4* from a 2μ plasmid were used. None of the Ssa constructs counteracted poly-Q toxicity in the isogenic [psi$^-$ PIN $^+$] and [PSI $^+$ pin $^-$] background (data not shown). C. Ssa1 and Ssa2 do not counteract poly-Q toxicity when expressed from *SSA2* promoter. Medium used was selective for plasmids and contained galactose to induce the poly-Q constructs (-Ura-His/Gal + Raf). Plate was photographed after 8 days of incubation. Data for OT60 and GT490 was similar and is not shown. D. Chaperones Hsp26 and Hsp82 do not counteract poly-Q toxicity. Medium selective for plasmids and containing galactose was used to induce poly-Q and chaperone constructs. Plate is photographed after 10 days of incubation. Data for OT60 and GT490 was similar and is not shown. Typical representatives are shown. See materials and methods for procedural details and plasmids used.
Strains used: [PSI$^+$ PIN$^+$] (OT56) , [psi$^-$ PIN$^+$] (OT60) and [PSI$^+$ pin$^-$] (GT490)

Lindquist, 2001), while overproduction of the other Hsp40 chaperone, Ydj1, has been shown to antagonize poly-Q aggregation in some yeast models (Muchowski et al., 2000). The Hsp40 chaperones Ydj1 and Sis1 did not affect Q103 toxicity at any detectable level in the [PSI^+] strains, but exhibited opposite effects on Q103 toxicity when overproduced in the [psi^- PIN^+] strain. Extra-copy of *YDJ1*, expressed from either the strong constitutive P_{GPD} promoter (Figure 4.2 A,C) or endogenous P_{YDJ1} promoter (Figure 4.4 A), increased Q103 toxicity. In contrast, extra copy of *SIS1*, expressed from either P_{GPD} (Fig. 4.2 B, D) or endogenous P_{SIS1} promoter (Figure 4.4 A), counteracted Q103 toxicity.

Figure 4.3 A shows different kinds of aggregates seen in [psi^- PIN^+] cells. Q103 forms large "clumps" in about 40% of the [psi^- PIN^+] cells, and either "dots" or both small clumps and dots in the rest of them. Fluorescence microscopy analysis confirmed that co-expression of Ydj1 with Q103 in the [psi^- PIN^+] background increased proportion of the endocytosis-defective cells with large Q103 clumps (Figure 4.3 B). Likewise, proportion of the Q103 protein which is insoluble and pelletable at 10,000 g was increased in the presence of excess Ydj1 (Figure 4.3 D). In contrast, the [psi^- PIN^+] cultures co-expressing Sis1 with Q103 exhibited a decrease in proportion of the endocytosis-defective cells with large clumps, in comparison to those expressing Q103 alone. This was accompanied by an increase in proportion of the cells with dots, or with both dots and small clumps (Figure 4.3 C), and by decrease in proportion of the Q103 protein pelletable at 10,000 g (Figure 4.3 E). Neither excess Ydj1 nor excess Sis1 affected total levels of Q103 (data not shown). Excess Sis1 did not lead to any detectable loss of [PIN^+], confirming that effect of Sis1 is not due to loss of a prion. Taken together, these data suggest that excess Ydj1 exacerbates toxic effect of Q103 by increasing size of Q103 aggregates, while excess Sis1 counteracts toxicity by decreasing aggregate size.

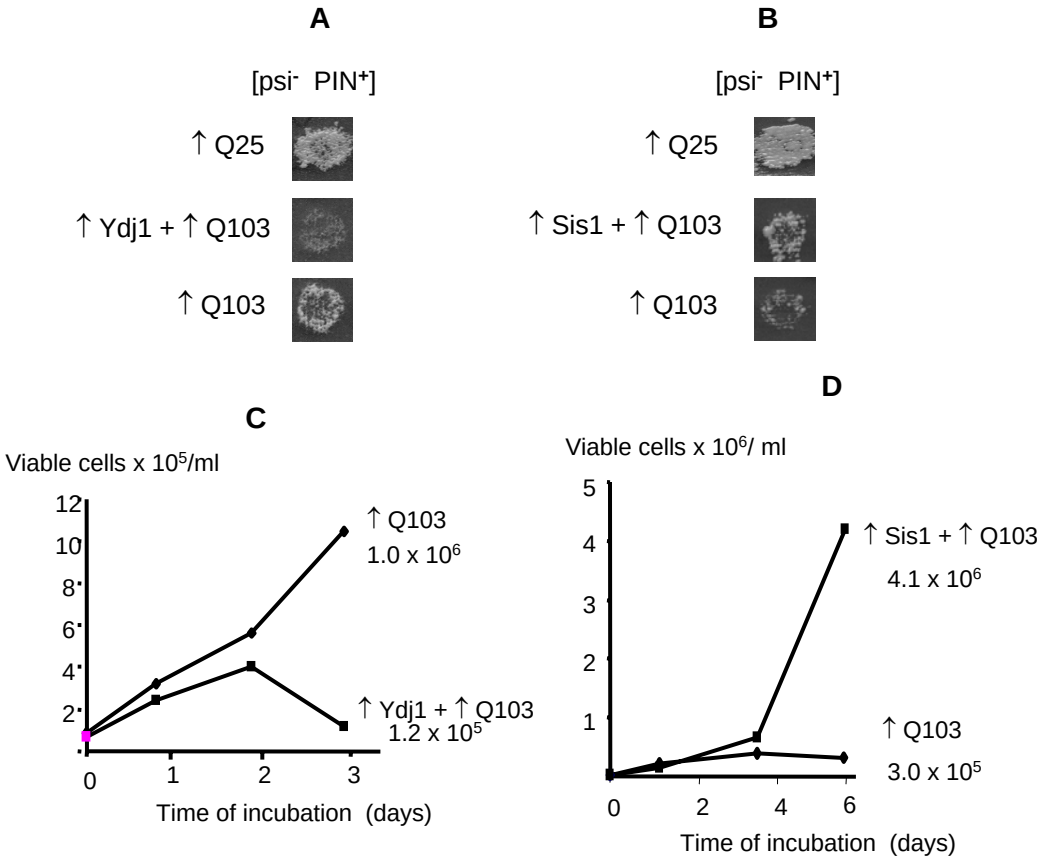


Figure 4.2 Effects of Hsp40 chaperones on Q103 toxicity
A and C - Excess Ydj1 increases Q103 toxicity in [psi$^-$ PIN$^+$] background. B and D – Excess Sis1 decreases Q103 toxicity in [psi$^-$ PIN$^+$] background. A and B – Plate Assay: The –Ura-His/ Gal (Ydj1) and –Ura-Trp/ Gal (Sis1) medium were used to select for plasmids and to induce the poly-Q constructs. Plates were photographed after 7 days of incubation (second passage) for Ydj1 and after 3 days of incubation (second passage) for Sis1. Differences in media composition reflects the difference in Q103 control. No effects of Ydj1 or Sis1 on toxicity were detected in the [PSI$^+$] background (not shown). C and D – Quantitative Assay. Average numbers from 2 repeats of each of two independent transformants are shown in each case. Cultures were grown in liquid respective selective medium containing galactose and raffinose. Standard deviation did not exceed 27% of the value for each time-point. Differences between Q103 cultures and cultures expressing Ydj1 or Sis1 were statistically significant after 3 days and 6 days respectively. Differences in media composition reflects the difference in Q103 control and is consistent with plate assay. Q103 control grows much more slower in –Ura-Trp/Gal than in –Ura-His/Gal medium.Strains used same as mentioned in legend 4.1.

Plasmids: Ydj1: pRSYDJ1; Sis1: pTVSIS1

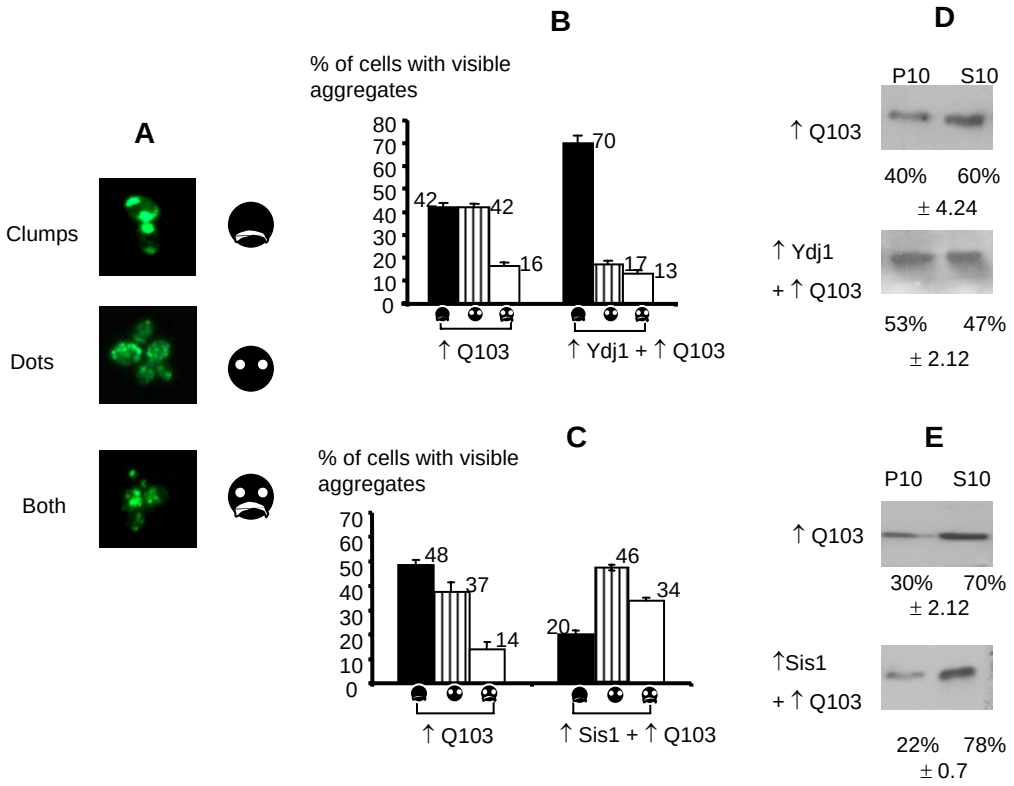


Figure 4.3 Effects of Hsp40 chaperones on Q103 aggregates
A.Types of aggregates observed in Q103 overexpressing cells. [psi$^-$ PIN $^+$] cultures were grown in liquid –Ura-His/ Gal + Raf medium to induce poly-Q. Three distinct classes of cells with aggregates were observed – 1) cells containing large clumps, (☹) 2)cells containing dot-like structures (😐) and 3)cells containing both clumps and dots (😣). B and C - Excess Ydj1 increases proportion of cells with Q103 clumps while excess Sis1 decreases it. Error bars indicate standard deviation. Cultures were grown in liquid –Ura-His/Gal + Raf (Ydj1) and in –Ura-Trp/Gal + Raf (Sis1) overnight. D and E – Excess Ydj1 decreases proportion of soluble Q103 (D) and excess Sis1 decreases it (E). Proteins were isolated in the [psi$^-$ PIN $^+$] strain after 24 hours of growth in synthetic Gal + Raf medium selective for plasmids and fractionated as described in Materials and Methods (Chapter 2). Pellet (P) and Supernatant (S) fractions at 10,000 g are shown. Proportion of Q103-GFP protein in P and S are determined by densitometry. For strain and plasmids used, see 4.2 Figure legend.

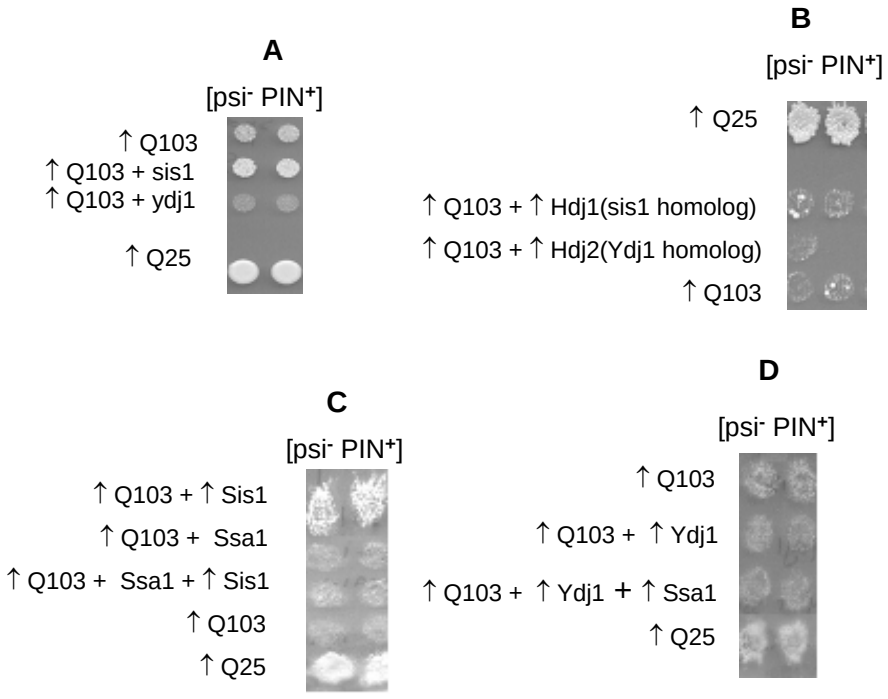


Figure 4.4 Modulation of Ydj1 and Sis1 effects on Q103 toxicity
A. Single copy Ydj1 increases and single copy Sis1 decreases Q103 toxicity in [psi- PIN+] strain. Medium used is –Trp-Leu/ Gal selective for plasmids and containing galactose to induce the poly-Q constructs. Plate was photographed after 8 days of incubation. Plasmids used: "↑ Q103 ": pYES-Q103trp, " Sis1" – pRS315Sis1, "Ydj1"- pRS315Ydj1 . Control plasmid used is pRS315. B. Mammalian homolog of Ydj1 and Sis1 do not have any effects on Q103 toxicity. Medium used is selective for plasmids and contains galactose to induce poly-Q constructs (-Ura-Trp/Gal). Plate is photographed after 14 days of incubation. Plasmids used: "↑ Q103" – pYES2103Q, "↑ Hdj1 "-Hdj1424GPD; "↑ Hdj2" - Hdj2424GPD , "↑ Q25" – pYES225Q. Control plasmid used is pFL39. OT60 data is shown. GT490 and OT56 show similar results and is not shown. C. Ssa1 eliminates the Sis1's Q103 toxicity compensatory effect. Medium used is selective for plasmids and contains galactose to induce poly-Q constructs (-Ura-His-Trp/Gal).Plate is photographed after 7 days of incubation. Plasmids used "↑ Q103 ": pYES2-103Q, " Sis1" – pTVSIS1,"Ssa1": pN2 . Control plasmids used are pRS316GAL, pLA1 and pFL39. D. Ssa1 does not have any effect on Ydj1's ability to increase Q103 toxicity. Medium used is same as in 4.4C. Plate is photographed after 3 days (second passage).
Plasmids used are "↑ Q103 ": pYES-Q103trp, "Ydj1"- pRSYDJ1, "↑ Ssa1": pGALSSA1.
Strains used: [PSI+ PIN+] (OT56) , [psi- PIN+] (OT60) and [PSI+ pin-] (GT490)

Since Hsp40 are considered to be co-chaperones of Hsp70, we checked the effects of Hsp70-Ssa1 and Hsp40-Sis1 or Hsp40-Ydj1 simultaneously. Ssa1 did not change the already observed effects of Sis1/Ydj1 on poly-Q toxicity (Figure 4.4 C, D). We also checked the effects of mammalian homologs of Sis1 and Ydj1 known as Hdj1 and Hdj2 respectively on poly-Q toxicity. Neither showed any observable effect irrespective of the prion background (Figure 4.4 B).

Effect of mutations in the peptide-binding domain and C-terminal truncations of *SSB1* on drug sensitivity

The [psi$^-$ PIN$^+$] and [PSI$^+$] *ssb1,2*Δ strains, GT157 and GT146 respectively were transformed with *SSA-SSB* chimeras (Figure 4.5) and checked for compensation of Par^s, Hyg^{s,}, and Ghc^s phenotypes by K. Allen (Chernoff Lab). Her studies showed that that the peptide-binding domain of Ssb in combination with at least one other domain of Ssb origin were capable of compensating for sensitivity to paromomycin and GuHCl in our strains background (Allen et al., 2004), while only complete Ssb was compensating for Hygromycin sensitivity (Allen et al., 2004). In this chapter, we extend this study by testing several point mutations in the peptide binding domain and C-terminal truncations of Ssb (Pfund et al, 2001) on drug sensitivity in the [psi$^-$ PIN$^+$] and [PSI$^+$] *ssb1,2*Δ strains mentioned above. Full length *SSB1*, point mutant alleles of *SSB1*: *SSB1-M409A*, *SSB1-V432S*, *SSB1-T443A, SSB1-F444S, SSB1-F444I*, compensated the drug sensitivity of *ssb1,2*Δ strains when tested on both complete medium and synthetic medium with all three drugs, Paromomycin, Hygromycin and GuHCl (Figure 4.6 A, B). Ssb1-V442F showed complete Hyg^s , while partial Par^s and Ghc^s on complete medium (Figure 4.6 A). On the contrary such an effect was not detected on synthetic medium (Figure 4.6 B). Ssb1-Δ488 showed drug sensitivity when tested on complete medium and synthetic medium, with the exception of GuHCl on synthetic medium (Figure 4.6 A, B). Ssb1-Δ499

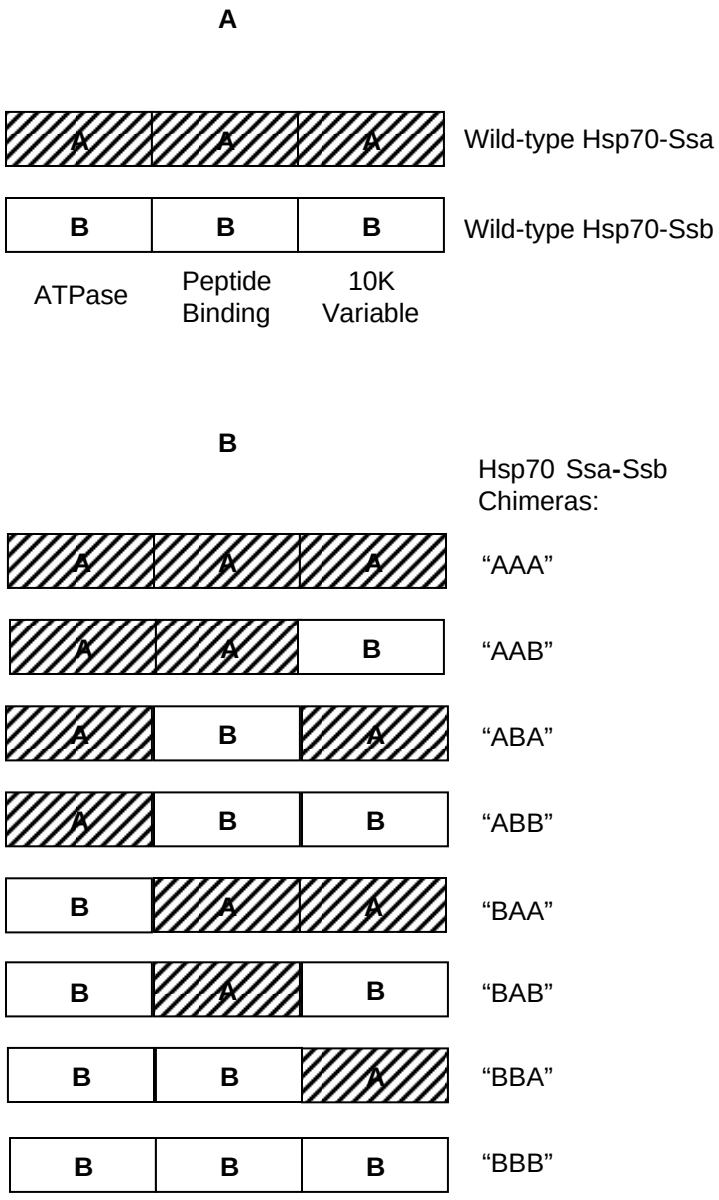


Figure 4.5 Schematic representation of Hsp70 Ssa-Ssb chimera proteins
A. Hsp70 family proteins share three distinct functional domains: The N-terminal ATPase domain, the 18-kDa peptide-binding domain, and the C-terminal 10-kDa variable domain. B. Recombinant DNA technology was used to generate two sets of expression vectors encoding Hsp70 Ssa-Ssb protein chimeras (James et al., 1997; Pfund et al*.,* 2001). One set was placed under the *TEF1* promoter while the other set was placed under the *HSP70* promoter corresponding to the source of the ATPase domain. In this study, each plasmid is designated according to the promoter or the plasmid backbone, and the chimeric combination it encodes. For example, pTEF-ABA and pRS316K-ABA encode ABA chimeric proteins under the *TEF1* promoter and *HSP70-SSA* promoter, respectively.

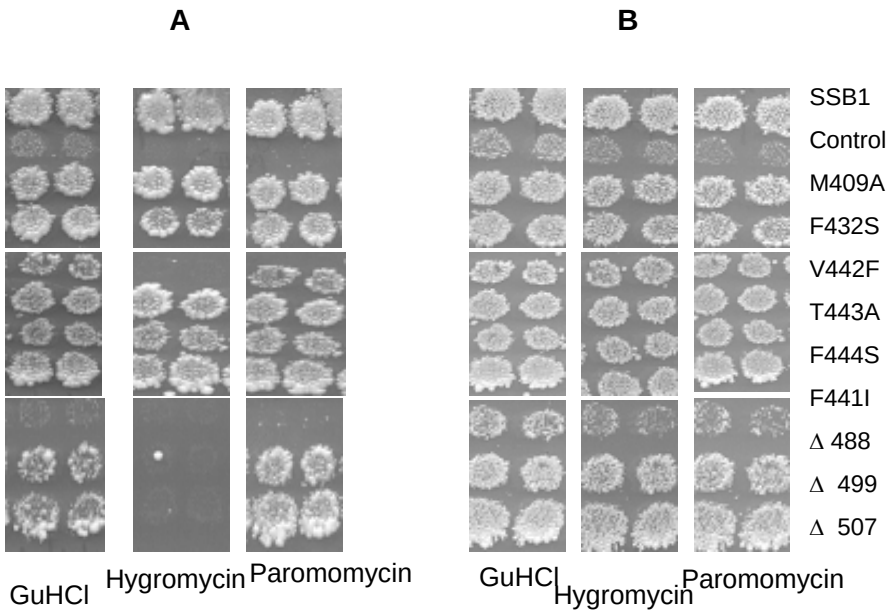


Figure 4.6 Drug sensitivity of *SSB* mutants in *ssb1,2Δ* [PSI$^+$ PIN$^+$] on A. complete medium YPD and B. synthetic medium SD + 13 Transformants were velveteened onto medium containing additions of drugs as mentioned in materials and methods. Ssb-V442F causes complete sensitivity to Hygromycin and partial sensitivity to GuHCl and paromomycin seen at detectable levels only when tested on YPD (complete medium). Ssb1-Δ488 shows sensitivity to all three chemicals on YPD medium while only with hygromycin and paromomycin on synthetic medium. Ssb1-Δ499 and Ssb1-Δ507 shows sensitivity to hygromycin only on YPD medium. [psi$^-$ PIN$^+$] data is similar and not shown.
Strains used: *ssb1,2Δ* [PSI$^+$ PIN$^+$]: GT146 , *ssb1,2Δ* [psi$^-$ PIN$^+$]: GT157
Plasmids used: pRS316 based *SSB1* wild-type allele, mutant alleles and truncations given in materials and methods.

and Ssb1- Δ507 showed Hyg^s only on complete medium, while these truncations could otherwise compensate drug sensitivity of other drugs on complete medium and synthetic medium (Figure 4.6 A, B). Presence or absence of [PSI$^+$] did not affect the final outcome.

Effect of mutations in the peptide binding domain and C-terminal truncations of *SSB1* on Hsp104's ability to cure [PSI$^+$]

Ssa proteins – member of the Hsp70 family are coinduced with Hsp104 during heat shock, stationary-phase growth, and sporulation (Kurtz et al., 1996; Werner-Washburne et al., 1987; Werner-Washburne et al., 1989). Ssa1 –member of the SSA family was shown to protect [PSI$^+$] from Hsp104-mediated curing (Newnam et al., 1999). On the other hand, Ssb1 overproduction increased [PSI$^+$] curing by excess Hsp104 (Chernoff et al., 1999) and infact Ssb1 overproduction by itself could also cure [PSI$^+$] (Chacinska et al., 2001). Here we wanted to study if the ability of Ssb1 to increase [PSI$^+$] curing by excess Hsp104 is affected by any of the mutations in the peptide-binding domain or C-terminal truncations. None of the mutations/truncations in Ssb1 affected [PSI$^+$] suppression by itself when checked in [PSI$^+$] strains GT146, GT202 and GT203 (data not shown). Peptide-binding domain of Ssb1 was shown to be essential for its activity (Pfund et al., 2001). However none of the mutations in this domain affected Ssb's ability to cure [PSI$^+$] by excess Hsp104 (Figure 4.7). Infact, Ssb lacking the complete variable C-terminal domain (Ssb1-Δ507) interfered with its ability to increase [PSI$^+$] curing by excess Hsp104 (Figure 4.7) and the Ssb lacking the variable C-terminal domain and the C-terminal portion of the peptide binding domain containing the two last β strands 7 and 8 (Ssb1-Δ488) partially affected Ssb's ability to cure [PSI$^+$] (Figure 4.7). Strangely enough (Ssb1-Δ499) lacking only the last β strand 8 of peptide binding domain

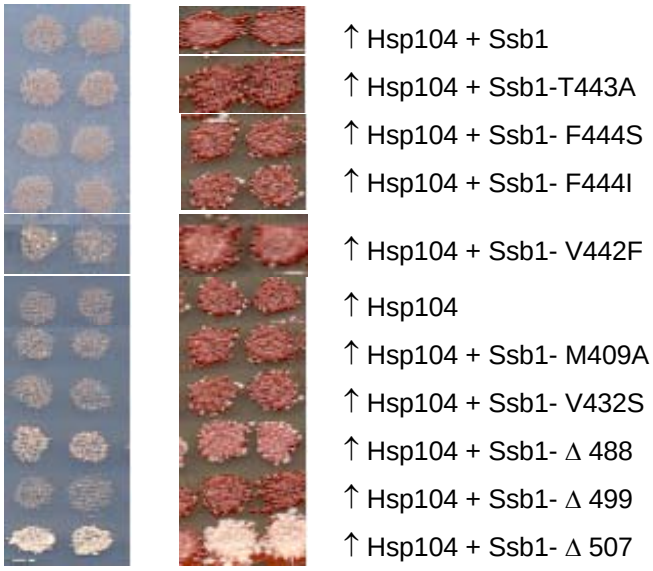


Figure 4.7 Effect of mutations in peptide binding domain and C-terminal truncations of Ssb on Hsp104's ability to cure [PSI$^+$]
Transformants were grown on medium selective for plasmids (-Ura-His) velveteened onto -Ura-His/Gal to induce the Hsp104 construct by growing for about 3 days and then velveteened onto YPD (color) and –ura-his-ade (suppression) to check for [PSI$^+$]. –Ade plate was photographed after 10 days of incubation and YPD plate was photographed after 2 days of growth at 30°C and 2 days of refrigeration at 4°C for color development. Ssb1-Δ488 followed by Ssb1-Δ507 affect Hsp104's ability to cure [PSI$^+$].
Strain used: OT56 [PSI$^+$ PIN$^+$]

and the complete C-terminal domain did not compromise Ssb's ability to cure [PSI$^+$] (Figure 4.7).

Effect of chimeric *SSA-SSB's* on [PSI$^+$]^{PS}

Effect of Hsp70-Ssa and Ssb was also studied on [PSI$^+$]^{PS}. Ssa does not affect suppression by [PSI$^+$]^{PS} and Ssb cures [PSI$^+$]^{PS}. Ssa was shown to increase *Saccharomyces* [PSI$^+$] suppression (Allen et al., 2004) while Ssb was shown to increase Hsp104's ability to cure *Saccharomyces* [PSI$^+$]. Because *Pichia-Saccharomyces* [PSI$^+$] is very unstable, heterogeneity in color was observed. But in general in majority of transformants, when the chimeric protein contained any two domains of Ssb1- either the ATPase domain or the peptide-binding domain, or the variable domain, the protein had a curing effect on *Pichia* [PSI$^+$] (Figure 4.8 A). On the other hand, when the chimeric protein contained any two domains of Ssa1, it did not affect suppression by *Pichia-Saccharomyces* [PSI$^+$] (Figure 4.8 A).

When the mutations in the peptide-binding domain and C-terminal truncations of Ssb1 were tested with *Pichia-Saccharomyces* [PSI$^+$], two mutations F444S and F432S affected [PSI$^+$]^{PS} to a larger extent than the wild-type Ssb1 alone. Strangely enough, the curing effect of Ssb1 on [PSI$^+$]^{PS} were not strong enough to see effects of mutations affecting the curing (Figure 4.8 B).

Interaction between Ssa/Ssb and *Saccharomyces/Pichia* Sup35

We employed two-hybrid approach to test if an interaction between Sup35 from either *S cerevisiae* or *P methanolica* and Ssa or Ssb exists . Interaction between Sup35Sc and Ssa was seen as growth on –Ade medium by the activation of *GAL2-ADE2* reporter (Figure 4.9 A). Ssa did not show 2-hybrid interaction with Sup35Pm (Figure 4.9 A). This interaction was confirmed by the activation of another reporter

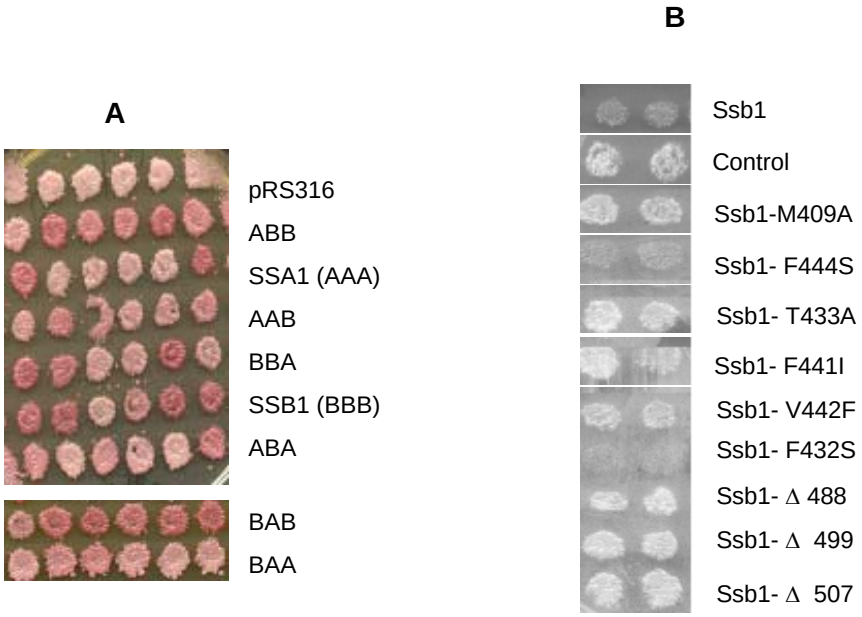


Figure 4.8 Effect of Ssa and Ssb on [PSI$^+$]^{PS}
A. Effect of chimeric Ssa-Ssb proteins on [PSI$^+$]^{PS}. Medium used is –Ura selective for the plasmid. The transformants were velveteened onto YPD to check for the presence of [PSI$^+$] by color. Lot of heterogeneity is observed in terms of color partly because [PSI$^+$]^{PS} is unstable. *SSA1* does not affect suppression by [PSI$^+$]^{PS} and *SSB1* cures [PSI$^+$] ^{PS} in contrary to *SSA1* increasing suppression by *Saccharomyces* [PSI$^+$] (Allen et al., 2004) and *SSB1* only increasing curing effect by excess Hsp104 but not by itself (Chernoff et al., 1999). No particular domain of *SSA* or *SSB* seems to be responsible for the shift between [PSI$^+$] maintenance and curing. See materials and methods for details on what the chimeric ABB etc stand for. Note that this chimeric proteins were expressed under their own promoter. Data for chimeric proteins expressed under *TEF* promoter did not show sharp color differences and is not shown. B. Effect of peptide-binding domain mutations in *SSB1* on [PSI$^+$]^{PS} suppression. The transformants were grown on medium selective for plasmid (-Ura) and velveteened onto –Ura-Ade to check for suppression. Plate is photographed after 6 days. All the mutations and truncations in Ssb1 do not affect suppression by *Pichia* [PSI$^+$] ^{PS} with the exception of Ssb1-F444S and Ssb1-F432S that along with Ssb1 cure [PSI$^+$]^{PS}

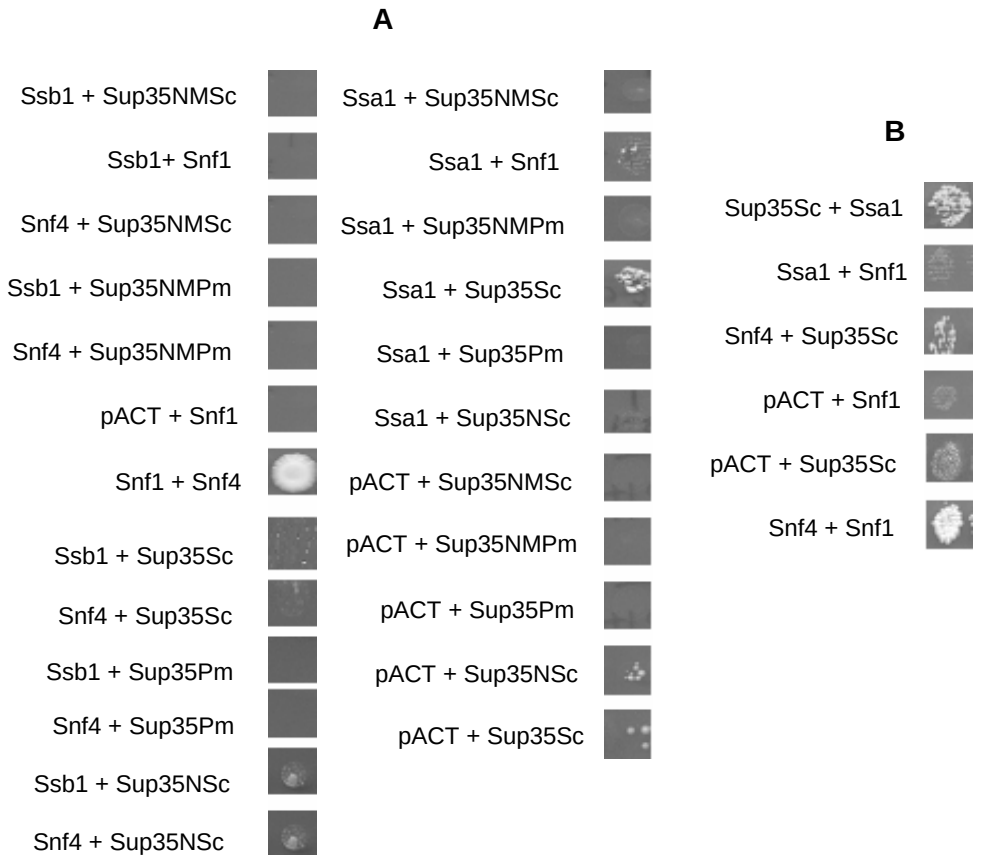


Figure 4.9 Yeast two-hybrid assay to check for interaction between Ssa/Ssb and Sup35 of *Saccharomyces cerevisae (Sc)* and *Pichia methanolica (Pm)*
A. Activation of *GAL2-ADE2* detected as growth on –Ade indicating yeast two-hybrid interaction. Depicted is growth seen after 9 days of incubation a by spotting on medium selective for plasmids and lacking adenine (-Trp-Leu-Ade). Plasmid bearing the *GAL4* DNA-binding domain under the *ADH* promoter is *TRP1* marked and the plasmid (pACT) bearing the activation domain of *GAL4* under the *ADH* promoter is *LEU2* marked. Plasmids containing *SNF1* and *SNF4* are used as a positive control. Weak interaction between Ssa1 and *Saccharomyces* Sup35 is seen as papillatory growth compared to the strong growth seen by the interaction between Snf4 and Snf1. Sup35 alone also shows non-specific interaction seen as weak papillatory growth with pACT + Sup35 combination. B. Activation of *GAL1-HIS3* detected as growth on –His confirming yeast two-hybrid interaction. Depicted is growth on –Trp-Leu-His + 1mM aminotriazole seen after 5 days of incubation. See materials and methods on principle and working of yeast two-hybrid method and the plasmids and strains used. *SNF1, SUP35Sc,SUP35NSc,SUP35NMSc, SUP35NMPm, SUP35Pm* are all fused to the *GAL4* DNA-binding domain, while *SNF4, SSA1, SSB1* are fused to the activation domain of *GAL4.*

system *GAL1-HIS3* as growth on –His medium (Figure 4.9 B). Physical interactions in vitro and in vivo were seen between Sup35Sc and Ssa (Allen et al., 2004). However Ssb did not exhibit two-hybrid interaction with either the full length or N/NM portions of Sup35Sc or Sup35Pm.

Discussion

Do Hsp70/Hsp40 chaperones influence poly-Q toxicity?

Both yeast Hsp40 proteins influencing poly-Q toxicity, Sis1 and Ydj1, are thought to be co-factors of the Hsp70 chaperones of Ssa subfamily (Bukau and Horwich, 1998). Yeast Ssa1 was shown to suppress the formation of insoluble poly-Q aggregates in the yeast cells (Muchowski et al., 2000; Cummings et al., 2001). However, simultaneous depletion of the two members of Ssa subfamily, Ssa1 and Ssa2, decreased prion-dependent Q103 aggregation in our system (Meriin et al., 2002), and excess Ssa promoted rather than decreased aggregation of the yeast prion protein Sup35 in our hands (Allen et al., 2004; Newnam et al., 1999).

One could suggest that Hsp40 chaperones act on poly-Q aggregates via modulating activity of their Hsp70 interacting partners. However, our data show that only one of the 4 members of the *S. cerevisiae* Ssa subfamily, Ssa4, counteracts Q103 toxicity when overproduced. Moreover, antitoxic effect of Ssa4 could be seen clearly only in the yeast strains containing both prions, [PIN^+] and [PSI^+] simultaneously (Figure 4.1 B). Differential effects of various members of the Hsp40 and Hsp70 families on Q103 confirm that chaperone interactions with poly-Q aggregates are of highly specific nature, and suggest caution in interpreting the previous data where only some members of the family were studied. For example, effect of the *ssa1/2* deletion (Meriin et al., 2002) could be not due to elimination of Ssa1 and Ssa2 per se, but due to increased fraction of Ssa4

in the total Ssa pool. Interestingly, while all Ssa proteins act in the same direction on [PSI^+] (Allen et al., 2004), Ssa1 and Ssa2 differ from each other in their effects on the other yeast prion, [URE3] (Schwimmer and Masison, 2002). Further investigations are needed in order to establish molecular basis of the differential effects of Ssa chaperones on protein aggregates.

Differential effects of Hsp40 chaperones on poly-Q aggregation and toxicity

Yeast Sis1 and Ydj1, functionally distinct heat shock proteins of the Hsp40 family, are homologs of the human Hsp40 proteins Hdj1 and Hdj2, respectively. In the *Drosophila* model of a poly-Q disease, both proteins suppressed neurodegeneration but Hdj1 had a stronger effect than Hdj2 (Chan et al., 2000). Interestingly, overexpression of Hdj2 increased inclusion formation by Htt in the mammalian COS7 cells (Wyttenbach et al., 2000). Neither when tested however in yeast showed any effect on poly-Q toxicity (Figure 4.4 B) suggesting the requirement of a specific environment that cannot be created in yeast. Yeast Ydj1, a homolog of Hdj2, has previously been shown to suppress rather than increase formation of insoluble aggregates of polyQ-expanded exon 1 of Htt in yeast (Muchowski et al., 2000; Cummings et al., 2001). Effect of Sis1 on poly-Q aggregation in yeast has not previously been studied.

In our system, excess Sis1 decreased Q103 aggregate size (Figure 4.3 C, E) and counteracted Q103 toxicity (Figure 4.2 B, D), similar to the effect of its mammalian homolog Hdj1 in the other models. Intact Sis1 is required for the maintenance of [PIN^+] prion (Sondheimer et al., 2001), however excess Sis1 did not cure cells of [PIN^+], ruling out a possibility that anti-toxicity was due to loss of a prion. In contrast to Sis1, excess Ydj1 increased Q103 aggregate size (Figure 4.3 B, D), similar to the effect of its mammalian homolog Hdj2 at least in some assays (Wyttenbach et al., 2000), and increased Q103 toxicity (Figure 4.2 A, C). This agrees with the previous observation that

Q103 aggregation is decreased in the strain carrying *ydj1-159* mutation (Meriin et al., 2002). Interestingly, our preliminary data indicate that Sis1 and Ydj1 also differ from each other in regard to their effects on the yeast prion [PSI^+] (S. Müller, J. Patterson and Y. Chernoff, unpublished data). Differences between our results and previous reports on the effects of yeast Ydj1 on poly-Q (Muchowski et al., 2000; Cummings et al., 2001) are probably due to differences in the poly-Q constructs employed, yeast strains and/or prion compositions. For instance, our plate assay has not detected any effects of Hsp40s on poly-Q toxicity in the presence of both [PSI^+] and [PIN^+] prions simultaneously, possibly because toxicity was too strong to see an effect clearly (data not shown). As Hsp40s' effects observed in our [psi^- PIN^+] strain parallel observations in higher eukaryotic systems, one could argue that the yeast assay used in our work better reproduces patterns of interactions between poly-Q aggregates and chaperones in the homologous mammalian systems. The co-operative effect of Hsp40 and Hsp70 however could not be detected in this system.

Effect of Ssa-Ssb's on [PSI^+]^S and [PSI^+]^{PS}

The Ssa and Ssb Hsp70 families of *S cerevisiae* share 60% amino acid identity and reside in cytosol (Craig and Jacobsen, 1985) yet each have distinct, non-overlapping functions in vivo –Ssa proteins function in protein translocation into organelles and regulate the heat shock response (Craig and Jacobsen, 1985) while Ssb proteins are associated with translating ribosomes (Nelson et al., 1992). They have opposite effects on *Saccharomyces* [PSI^+] –Ssa favoring maintenance while Ssb favoring curing. Deletion of both Ssb-encoding genes, *Ssb1/2*Δ*,* causes sensitivities to some translational inhibitors. It was shown that constructs containing the peptide-binding domain and atleast one other domain of Ssb was able to complement some drug sensitivity (Allen et al., 2004), we tested which of the mutations in the peptide binding

domain and C-terminal truncations of Ssb is responsible for the drug sensitive phenotype. Interestingly, the C-terminal truncations had the most dramatic effect on drug sensitivity, while only one mutation in peptide-binding domain V442F showed drug sensitivity (Figure 4.6). Interestingly, the C-terminal truncation of Ssb1 affected ability of Hsp104 to mediate [PSI$^+$] curing implicating that the C-terminal domain of Ssb1 seems to be important in Ssb's function in relation to drug sensitivity in our strain prion background and [PSI$^+$] curing. Surprisingly the chimeric protein BBA (containing Ssa's C-terminal variable domain) does not affect Ssb's function in showing drug resistance (Pfund et al., 1997).

Excess Ssa1 was shown to increase [PSI$^+$] formation by overproduced Sup35N from the same species (Allen et al., 2004), and also by heterologous Sup35NM protein derived from an evolutionarily distant yeast, *Pichia methanolica* (K Allen, unpublished results). It was shown that construct carrying all three domains of Ssa (AAA) was able to increase non-sense suppression in the weak [PSI$^+$] strain OT55, (Allen et al., 2004), we observed that the construct with any two Ssa domains (AAB, ABA, BAA) were able to maintain [PSI$^+$]^{PS} just as with AAA. Infact peptide-binding domain of Ssb1 was shown to be necessary and sufficient for the inhibition of [PSI$^+$] mediated suppression (Allen et al., 2004), we observed that any two domains of Ssb1 (ABB, BAB) were equally effective as BBB in curing [PSI$^+$]^{PS}. The construct BBA showed 50% curing of [PSI$^+$]^{PS}, and the unstable nature of [PSI$^+$] ^{PS} made it difficult to intepret this result. Our data showing a two-hybrid interaction between *Saccharomyces* Sup35 and Ssa goes hand in hand with the observation that Ssa co-localized with Sup35-GFP clumps in vivo (Allen et al., 2004) and it was also capable of interacting with Sup35NM in the affinity chromatography assays in vitro (Allen et al., 2004).

CHAPTER 5

CHARACTERIZATION OF THE *AQT* STRAINS

Background

Ubiquitination and QN-rich protein aggregates

During the past decade, it has become apparent that a set of ostensibly unrelated neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's and prion diseases, that involve protein misfolding and aggregation and which result in inclusion bodies and other aggregates within cells are linked to the Ubiquitin (Ub)-proteasome system (Tran and Miller, 1999; Mayer, 2003; Ciechanover, 2003). This link is because of the fact that these aggregates often contain ubiquitin, which is the signal for proteolysis by the 26S proteasome. The link between the ubiquitin-proteasome system and neurodegeneration has been strengthened by the identification of disease-causing mutations in genes coding for several ubiquitin-proteasome pathway proteins in Parkinson's disease (reviewed in Ross and Pickart, 2004). It has been suggested that protein aggregates can impair the function of the Ub-proteasome system (Bence et al., 2001).

The Ub-proteasome pathway regulates a wide variety of biological processes (Hershko and Ciechanover,1998). The degradation of abnormal or damaged proteins occurs, at least in part by the Ub-proteasome pathway. Proteins targeted for degradation via this pathway require conjugation of Ub through an isopeptide bond joining the C-terminal glycine of Ub to a side-chain amino group of lysine (K) in the target protein. Ub polymerization occurs by linkage of the K at position 48 of the proximal Ub to the C-terminal glycine of another Ub. This reaction requires the sequential action of three enzymes: Ub-activating enzymes (E1), Ub-conjugating enzymes (E2) and/or Ub-ligase

(E3) (Hershko and Ciechanover,1998). Ubiquitination is a reversible process facilitated by Deubiquinating (DUB) enzymes that disassemble Ub-conjugates releasing free Ub (Wilkinson, 2000). Ub-substrates bind to the 19S regulatory particle of 26S proteasome, are unfolded and translocated to the 20S proteasome core, where protein is degraded by proteinases (Finley, 2002).

Depletion of Ubp6 and Doa4 (DUBs) that result in Ub depletion has been shown to cause defects in phenotypic expression and de novo formation of the yeast prion [PSI^+] (Chernova et al., 2003). K. Allen (Chernoff lab) in her thesis work also found that the deletion of one of the major Ub-conjugating enzymes, Ubc4, results in an increase in spontaneous [PSI^+] formation. She also saw that excess Sup35/Sup35N related toxicity in [PSI^+] cells was reduced in *ubc4Δ* cells while *ubc4Δ* cells expressing Htt-Q103 showed intense papillation. Recall that Q103 toxicity is seen in the wild-type yeast cells containing endogenous prions (Meriin et al., 2002 and this study). The papillations indicated that a change has occurred in a subset of cells that enabled them to overcome Q103 toxicity. Here, we present a detailed characterization of such mutants.

Materials and methods

Yeast strains

Yeast strains that are unique to this study are described below. Other commonly used strains are described in Chapter 2, and Table 2.1. GT349 is *MAT*a *ubc4Δ::HIS3* derivative of GT81-1C [PSI^+ PIN^+]. GT386 is a [psi^- PIN^+] derivative of GT349 obtained by Hsp104 treatment (pGAL104). GT387 is a [psi^- pin^-] derivative of GT349 obtained after 3 successive passages on 5mM GuHCl. GT573, GT574 and GT575 are *AQT-2*, *AQT-7* and *AQT-9* strains respectively. These are GT349 [PSI^+ RNQ^+] *ubc4Δ* papillae. GT675,

GT676 and GT677 are [psi$^-$ PIN$^+$] versions of GT573, GT574 and GT575 respectively. They were obtained by Hsp104 treatment (pLH105). GT769, GT770 and GT771 are [psi$^-$ pin$^-$] versions of GT573, GT574 and GT575 respectively obtained after GuHCl treatment (see above). In cases, where [psi$^-$] is made from [PSI$^+$], [psi$^-$] status is verified by the absence of growth on –ade medium (see materials and methods for more details on prion detection). GT385-13A is a *MAT*α *ubc4*Δ [psi$^-$ rnq$^-$] haploid meiotic spore clone obtained through the dissection of the GT385 diploid. UBC4$^+$/ubc4Δ diploids were obtained by mating GT81-1D with Q103 and empty plasmid transformants of GT573, GT574 and GT575. The Q103 and empty plasmid transformants of GT349 mated with GT81-1D served as a control for this experiment. These diploids were used to check *UBC4*$^+$ dependence for *AQT* toxicity. The Q103 (pYES2-103Q) transformants of GT573, GT574 and GT575 are strains GT580, GT582 and GT608 respectively. The empty plasmid (pRS316GAL) transformants of GT573, GT574 and GT575 are strains GT579, GT581 and GT607 respectively. The *ubc4*Δ / ubc4Δ diploid were obtained by mating GT385-13A with GT573, GT574 and GT575 Q103 and empty plasmid transformants. They are called GT532, GT534 and GT756/GT724 respectively. The diploid GT349 mated with Gt385-13A also called GT606 served as a control for this experiment. *AQT-2, 7* and *9* homozygous diploids were obtained by mating the seggregants of opposite mating type from *AQT* heterozygous diploids. Eg*: AQT-2* homozygous diploid GT726 is a cross between GT532-9B and GT532-9D. Diploid selection is done by transforming one partner with *LEU2* empty plasmid and the other with pYES2-103Q (*URA3*) and selecting on a –ura-leu medium. Similarly, *AQT-7* homozygous diploid GT727 is a cross between GT534-2A and GT534-2B. *AQT-9* homozygous diploid GT728 is a cross between GT724-3B and GT724-3D. To conduct the Allelism experiment, *AQT-2/7*, *AQT-2/9* and *AQT-7/9* diploids were made. GT729 is a cross between poly-Q transformant of

GT573 and GT534-2B (*AQT-2/7*), GT741 is a cross between poly-Q transformant of GT573 and GT724-3B (*AQT-2/9*) and GT730 is a cross between poly-Q transformant of GT575 and GT534-2B (*AQT-7/9*). The strain GT784-8C, [PSI^+ PIN^+] MAT α *ubc4Δ rnq1Δ* is a haploid meiotic spore clone obtained from the diploid GT784. GT818 is the [psi^- pin^-] derivative of GT784-8C obtained by 5mM GuHCl treatment. Some of the strains mentioned were were made by undergraduate student Charles Kubicek (Chernoff Lab).

Plasmid constructs

The commonly used plasmids are listed in Chapter 2. Plasmids unique to this study are described here. The centromeric *LEU2* based genomic library plasmids used were p366 (control) and p366 library pools #1, #2, #3 and #4.

Diploid selection

Diploids were made by mixing two haploid strains of opposite mating type ie one parent has A mating type and the other has α mating type. This was done on complete medium like YPD. YPD was incubated at 30°C for a day and then some cells were taken from the patch and streaked out on selective medium. To ease the selection of diploids usually one parent was transformed with *LEU2* based plasmid and the other with a *URA3* plasmid. The diploids were then selected by growth on –ura-leu. Note that this was possible with only if the strains are auxotrophs for that particular aminoacid. The plasmids were chosen depending upon what the diploid was used for. Eg polyQ (*URA3*) plasmid was used while making diploids if they were to be checked for anti-toxicity or empty plasmid (*URA3*) was used if the diploid was to be used for other purposes. In case where diploid selection by this means was not possible, patch was streaked out on YPD again, single colonies were selected, patched and mated to strains OT37 (α *his4*

lys2) and OT38 (a *his4 lys2*) kindly provided by S. Liebman. Diploids cannot mate with these strains while haploids can. All the strains used in the experiment are from GT81 series that are lys auxotrophs. So diploid selection is most cases was to mate the single colonies to OT37 lawn or OT38 lawn on YPD. Incubate the plate for a day or two at 30°C. Velveteen the plate onto SD + lys. Single colonies that were haploid depending upon their mating type will show growth either on OT37 or OT38 lawn while the diploid single colonies will not show growth on either plate.

Chromosomal DNA isolation for preparing the *AQT* DNA library

QIAGEN Genomic-tip 500/G kit was used . It isolates upto 100μgm high molecular weight DNA. Advantages of using this kit is that the procedure is very gentle and results in negligible DNA shearing. DNA purified with Qiagen Genomic-tips is sized up to 150 kb with an average length of 50-100kb. DNA is free of all contaminants like RNA, protein and other metabolites and has A260/A280 ratios between 1.7 and 1.9.

Preparation of chromosomal DNA for pulse-field electrophoresis

Agarose embedded yeast DNA was prepared using the BioRAD Kit. Plugs were loaded onto a pulse field certified agarose gel (1.2%) at 6V/cm gradient. The samples were run for 42hrs to get a good separation between 600kb and 1Mb.

Testing of *RNQ1* –likely candidate gene

The [psi$^-$ PIN$^+$] *ubc4Δ* strain and the *AQT* [psi$^-$ PIN$^+$] *ubc4Δ* strain were crossed with [psi$^-$ pin$^-$] *ubc4Δ* rnqΔ (control) respectively. If *AQT* = *rnq1** all 4 spores were expected to be non-toxic and if *AQT* is not rnq1* than diff types of tetrads were expected (2:2, 3:1 and 4:0). While the control cross was expected to give 2 RNQ$^+$(toxic): 2 rnqΔ

nontoxic. From the 8 mutant tetrads tested, 3 show 4:0 anti-toxicity:toxicity, 3 show 3:1 and 2 show 2:2. Thus this shows that *AQT* mutation is not in the *RNQ1* gene. The wild-type cross showed 2:2 anti-toxicity: toxicity as expected.

Results

Note: *AQT* strains are in the *ubc4Δ* background and these strains are [PSI^+ RNQ^+] unless otherwise stated.

Effect of *ubc4Δ* on Q103 toxicity

The poly-Q extended (Q103) exon 1 of human huntingtin fused to GFP is toxic when expressed under galactose-inducible promoter in the [PSI^+ RNQ^+] $UBC4^+$ background but when expressed in cells in which Ubc4 is deleted (*ubc4Δ*) showed numerous papillations indicating that a subset of cells were able to counteract Q103 toxicity (Figure 5.1 A). 2 papillae from 12 independent *ubc4Δ* transformants were colony-purified and re-tested along with the original transformants from which they emerged for the anti-toxicity phenotype. Individual papilla was then re-tested for anti-toxicity. Q103 plasmid was lost and then re-introduced to test the anti-toxicity. Presence of [PSI^+] and [PIN^+] was tested from the 3 re-transformed papillae that continued to show anti-toxicity and were designated as " *AQT*" strains (*AQT-2, AQT-7 and AQT-9*) and further investigated (Figure 5.1 B). Q103 toxicity in yeast depends on the presence of [PSI^+] (Chapter 3) and [PIN^+] (Meriin et al., 2002). So it was important to check that this anti-toxic papillae emerging were simply not due to loss of endogenous prions [PSI^+] and

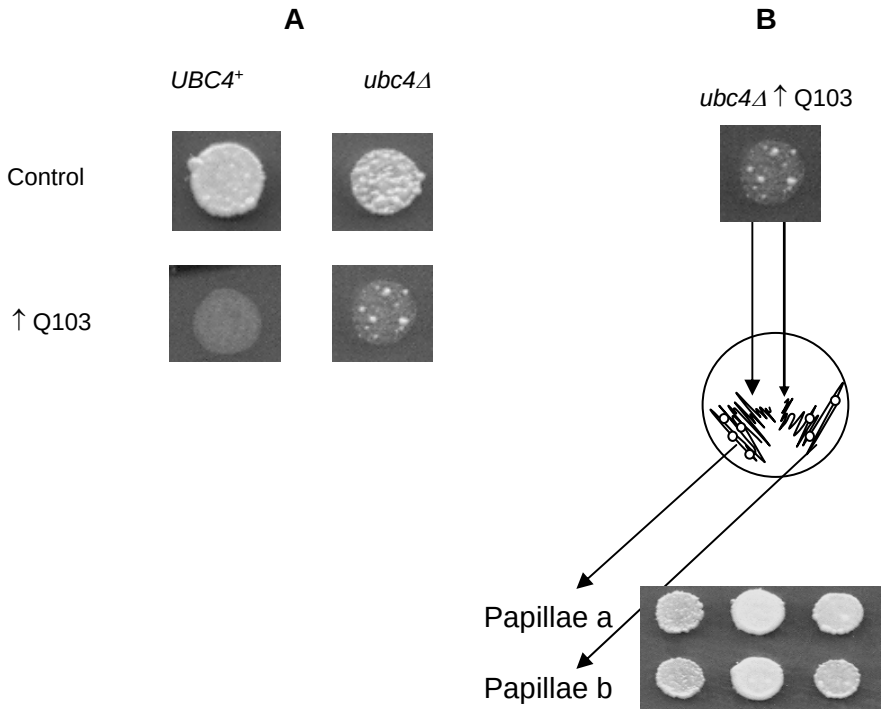


Figure 5.1 Origin of the *AQT* mutants
A. Papillation in *ubc4Δ* background in the presence of poly-Q: The [PSI$^+$ RNQ$^+$] strain, GT81-1C (*UBC4*$^+$) and GT349 (*ubc4Δ*) were transformed with pYES2-103Q encoding huntingtin Q103 and the empty vector control, pRS316GAL. Medium was selective for the plasmid (-Ura) and contained galactose (Gal) to induce the Q103 construct. Plates were photographed after 3 days of incubation. No differences in growth were seen on glucose (-Ura) medium (data not shown). Q103 toxicity is seen as growth inhibition on –Ura/Gal in the *UBC4*$^+$ background. Papillation is an indication that a subset of cells from the *ubc4Δ* background show anti-toxicity. B. " *AQT*" strains are papillae originating in the *ubc4Δ* background. 2 papillae from 12 independent *ubc4Δ* Q103 transformants were streaked out (only 2 are shown) and were checked for [PSI$^+$] loss, [RNQ$^+$] loss, plasmid retention and plasmid rearrangements (see text). 3 papillae that retained [PSI$^+$] and [RNQ$^+$] were designated as " AQT" strains (*AQT-2, AQT-7 and AQT-9*) and further investigated.

[PIN$^+$]. *AQT* strains showed the presence of [PSI$^+$] and [PIN$^+$]. (See Kim D. Allen's thesis for more details on obtaining *AQT* strains).

To confirm the observation that the Q103 anti-toxicity effect was dependent on the absence of Ubc4, we checked if Q103 toxicity could be restored by the introduction of Ubc4. Both extra-copy chromosomal *UBC4* (Figure 5.2 A) and multi copy episomal Ubc4 (Figure 5.2 B) were able to restore the Q103 toxicity seen in the *ubc4Δ* background with the exception that the *AQT-9* anti-toxicity phenotype was not completely overcome by the chromosomal copy of *UBC4* (Figure 5.2 A).

Characterization of the anti-toxicity phenotype of *AQT* mutants

Poly-Q Anti-toxicity effect of *AQT* strains was seen on solid medium containing galactose as growth which is more of a qualitative nature. We carefully analyzed this effect by conducting the experiment in liquid medium in a more quantitative manner. The *AQT-2* strain overexpressing Q103 grew better than its *ubc4Δ* isogenic strain and *UBC4*$^+$ isogenic strain (Figure 5.3 A). The growth of *AQT* strains with the empty plasmid was better than its *ubc4Δ* isogenic strain but lesser than its *UBC4*$^+$ isogenic strain (Figure 5.3 A) implying that *AQT* strains in general grew better than their *ubc4Δ* isogenic control in selective synthetic medium. Suprisingly, the growth of *ubc4Δ* strain was better in the presence of Q103 than its absence but it remained lot lesser than the *AQT-2* strain expressing Q103 (Figure 5.3 A). Trends for *AQT-7* and *AQT-9* were similar and are not shown.

When the *AQT* strains were transformed with Q103 expressed under Copper inducible promoter and checked on medium containing copper to induce the constructs, these strains grew equally well as their control strains (Figure 5.3 B). When checked on

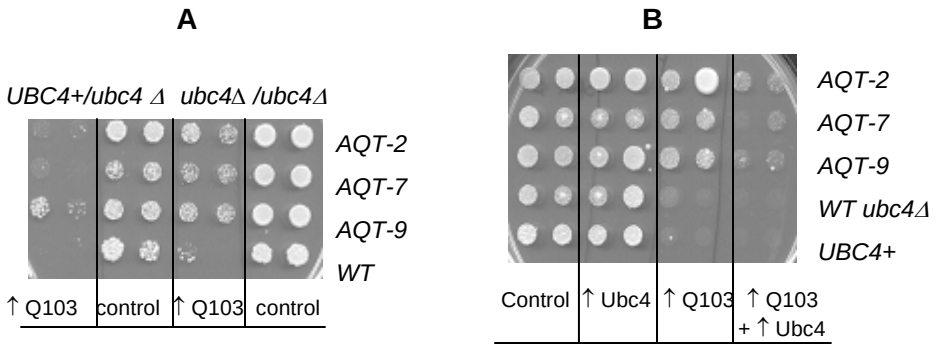


Figure 5.2 Anti-toxicity is *ubc4Δ* dependent
A. *UBC4+/ubc4Δ* diploids show Q103 toxicity while *ubc4Δ /ubc4Δ* diploids do not. The *AQT ubc4Δ* strain is mated with *UBC4+* isogenic strain to obtain *UBC4+/ubc4Δ* diploids. *The AQT ubc4Δ* strain is mated with *ubc4Δ* isogenic strain to obtain *ubc4Δ /ubc4Δ* diploids. See materials and methods for more details. Diploids were selected on –Ura-His and spotted onto galactose selective medium to induce the expression of Q103. Plates are photographed after 8 days of incubation on –Ura-His/Gal (second passage). B. Multi copy suppression of Q103 toxicity by *UBC4. AQT* strains -2,-7 and -9 GT573, GT574 and GT575 respectively along with the control strains GT349 (*ubc4Δ* Wild-type) and GT81-1C (*UBC4+* Wild-type) were transformed with control plasmids "Control"- pRS316GAL(*URA3*) and pFL39 (*TRP1*), "Ubc4"- pTRP-UBC4 and pRS316GAL;" ↑ Q103"- pYES2-103Q and pFL39 ;" ↑ Q103 + ↑ Ubc4" - pYES2-103Q and pTRP-UBC4 . Transformants were grown on –Ura-Trp/Glu selective for the plasmids and then spotted onto –Ura-Trp/Gal+ Raf to induce the constructs. Plates were photographed after 10 days of incubation.

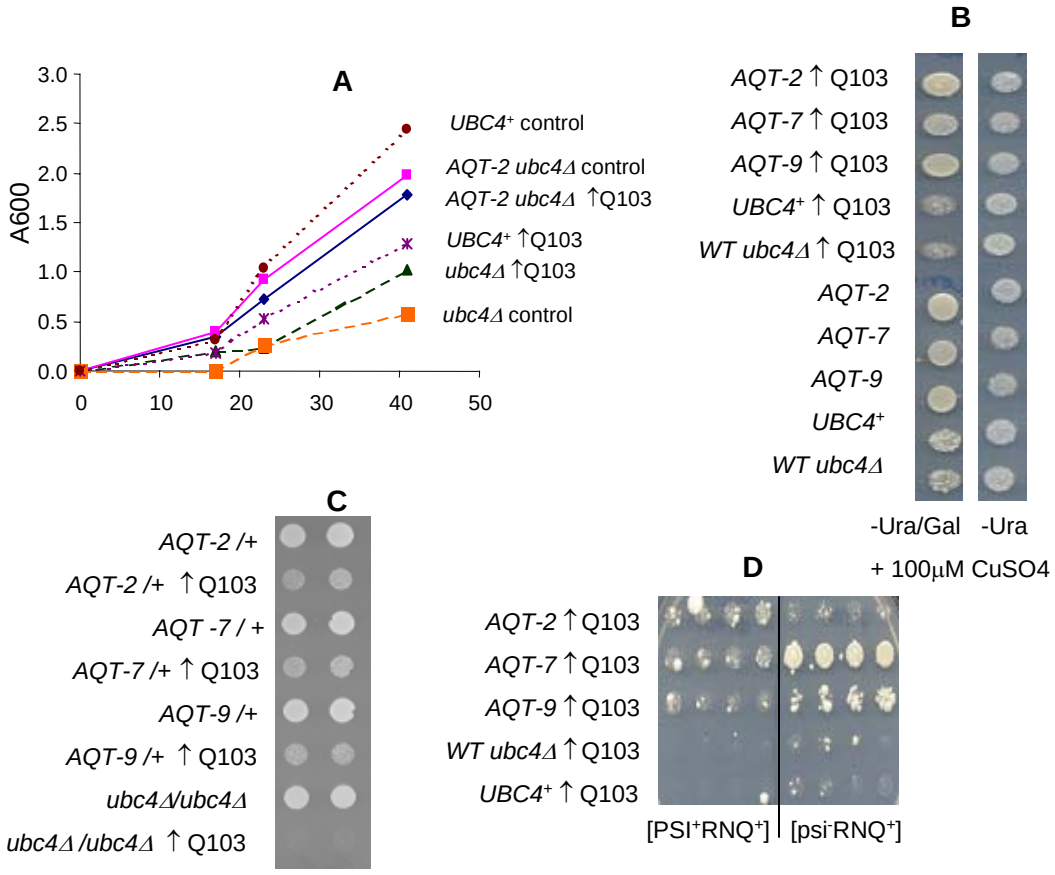


Figure 5.3 Anti-poly-Q toxicity phenotype of *AQT* mutants
A. Quantitative assay demonstrates Q103-anti-toxicity effect of *AQT* strains. *AQT* strains along with their controls were transformed with plasmids pYES2-103Q encoding Q103 and the control pRS316GAL. Transformants were grown in –Ura/Gal+ Raf to induce the Q103 construct. See materials and methods for more details. Data for *AQT-7* and *AQT-9* show similar trends and is not shown. B. *AQT* strains do not exhibit anti-Q103-toxicity phenotype when Q103 is expressed under *CUP* promoter. The *AQT* strains and the controls were transformed with the plasmid pMCUP-103Q-GFP encoding Q103 and the control plasmid pMCUP1. Transformants grown on –Ura were replica plated onto –Ura + 100μM CuSO4 with and without Gal. Plate was photographed after 8 days (second passage). C. Dominance of anti-toxicity in *AQT* mutants. *AQT* mutants along with the isogenic *ubc4*Δ control were mated with isogenic *ubc4*Δ control of the opposite mating type and were transformed with plasmids pYES2-103Q encoding Q103 and the control pRS316GAL. These *ubc4Δ /ubc4Δ* diploids were selected on -Ura-His. To check for anti-toxicity effect, they were spotted onto –Ura-His/Gal + Raf to induce the Q103 construct. Plate was photographed after 6 days of incubation. D. Effect of [PSI+] on anti-toxicity mediated by *AQT* strains. *AQT* strains along with their controls were compared to their [psi-] derivatives GT675, GT676, GT677 and their controls GT386 and GT159 for Q103 anti-toxicity. Plasmid used was pYES2-103Q encoding Q103. Spotted onto -Ura/Gal+Raf to induce the construct and plates were photographed after 10days. *AQT-7* shows an increase in anti-toxicity followed by *AQT-9* in the absence of [PSI+] while *AQT-2* shows no change in anti-toxicity irrespective of [PSI+].

copper containing galactose medium, growth of control strains with and without Q103 was impaired. The *AQT* strains with and without Q103 continued to show good growth (Figure 5.3 B). No differences in growth were detected on glucose medium irrespective of the presence of CuSO4 (data not shown). This data implies that the Q103 toxicity of control strains is galactose dependent. Differences in the expression levels of Q103 when expressed under galactose promoter and copper inducible promoter could account for this effect.

Next, we tested if the anti-toxicity trait is dominant or recessive. This was checked in diploids by mating each *AQT* strain with and without Q103 to its isogenic *ubc4*Δ strain. The diploids overexpressing Q103 continued to show anti-toxicity while the control wild-type *ubc4*Δ diploid overexpressing Q103 was not able to show growth as expected on galactose containing medium proving that the *AQT* Q103 anti-toxicity phenotype is dominant (Figure 5.3 C). Tetrad analysis of these diploids indicated that in most of the tetrads, spores showed 2:2 anti-Q103-toxicity: toxicity pattern of segregation (Table 5.1). 2:2 pattern of segregation indicates a mendelian trait. *AQT* phenotypes thus exhibit a mendelian (chromosomal) and not non-mendelian (epigenetic) pattern of inheritance as seen with prions.

Next, we checked if the mutation in all the three strains lied in the same or

Table 5.1 Tetrad analysis of *AQT* phenotype showing mendelian inheritance

Cross	**4:0**	**3:1**	**2:2**	**1:3**	**0:4**	**Total**
AQT-2 X *ubc4Δ*	0	0	5	0	2	7
AQT-7 X *ubc4Δ*	0	0	10	0	3	13
AQT-9 X *ubc4Δ*	0	0	8	0	0	8

different genes. In order to test that, *AQT-2/AQT-7*, *AQT-2/AQT-9* and *AQT-7/AQT-9* diploids were made by crossing one *AQT* strain with the other. These diploids were then checked for anti-toxicity. In most of the tetrads, spores showed 4:0 anti-Q103-toxicity: toxicity pattern of seggregation indicating that the mutation is in the same gene (Table 5.2). If mutations lied in different genes, different ratios were expected.

Table 5.2. Results of Allelism showing mostly 4:0 pattern of segregation of *AQT* anti-Q103- toxicity phenotype

Crosses	**Number of Tetrads tested**	**Anti-toxicity: Toxicity**
2/7	7	6 (4:0) 1 (3:1)
2/9	8	8 (4:0)
7/9	9	9 (4:0)

Since Q103 toxicity in yeast is dependent on the presence of prions, we further investigated if *AQT* strains that showed anti-polyQ toxicity also showed such dependence on [PSI^+]. [psi^- PIN^+] strains were transformed with Q103 along with their isogenic [PSI^+ PIN^+] strains. The absence of [PSI^+] increased the growth of *AQT-7* strain significantly and the growth of *AQT-9* strain only slightly. But *AQT-2* strain did not exhibit any increase in growth (ie increase in anti-toxicity) in the absence of [PSI^+] (Figure 5.3 D). The 3 strains differed in their ability to show anti-toxicity in the absence of [PSI^+] suggesting that differences could exist in the nature of mutation even though it appears to be affecting the same gene.

Characterization of growth of *AQT* strains

Growth of *AQT* mutants was monitored in complete medium to check if in general ie in the absence of poly-Q they grew better than their control strains. *AQT* strains grew better than its *ubc4Δ* isogenic control while *UBC4*$^{+}$ isogenic strain grew the best (Figure 5.4 A) just as seen previously in synthetic selective medium with strains transformed with empty plasmid (Figure 5.3 A).

AQT strains were also checked for temperature-sensitivity. Growth was monitored at different temperatures- 25°C, 37°C and 39°C on complete medium YPD. The growth of *ubc4Δ* wild-type control was affected at 39°C, while *AQT* strains continued to grow at 39°C along with its *UBC4*$^{+}$ wild-type strain (Figure 5.4 B). We also checked if prion background had any influence on temperature-resistance seen with the [PSI^{+} PIN^{+}] *AQT* strains. [PSI^{+} PIN^{+}], [psi^{-} PIN^{+}] and [psi^{-} pin^{-}] versions of *AQT* strains were tested. In case of *AQT-7* and *AQT-9*, the [psi^{-} PIN^{+}] versions showed temperature-sensitivity at 39°C (Figure 5.4 C). Thus *AQT-7* and *AQT-9* [psi^{-} PIN^{+}] strains behaved differently than *AQT-2* [psi^{-} PIN^{+}] strains in both temperature-resistance and dependence of anti poly Q toxicity on [PSI^{+}]. In the majority of cases, anti polyQ toxicity corresponded to temperature-resistance at 39°C. When checked if this temperature-resistance phenotype is dominant or recessive, the diploids same as used for checking if anti-poly-Q toxicity was dominant grew poorly at 39°C than the *AQT* haploid strains and their growth was not significantly better than its isogenic *ubc4Δ* diploid (Figure 5.4 D) indicating that the temperature-resistance phenotype is recessive.

Effects of the *AQT* mutations on Sup35/Sup35N toxicity

Overexpression of Sup35 or Sup35N (the prion forming domain of Sup35) in [PSI^{+}] cells is toxic to yeast cells (Chernoff et al., 1992; Ter-Avanesyan et al., 1993).

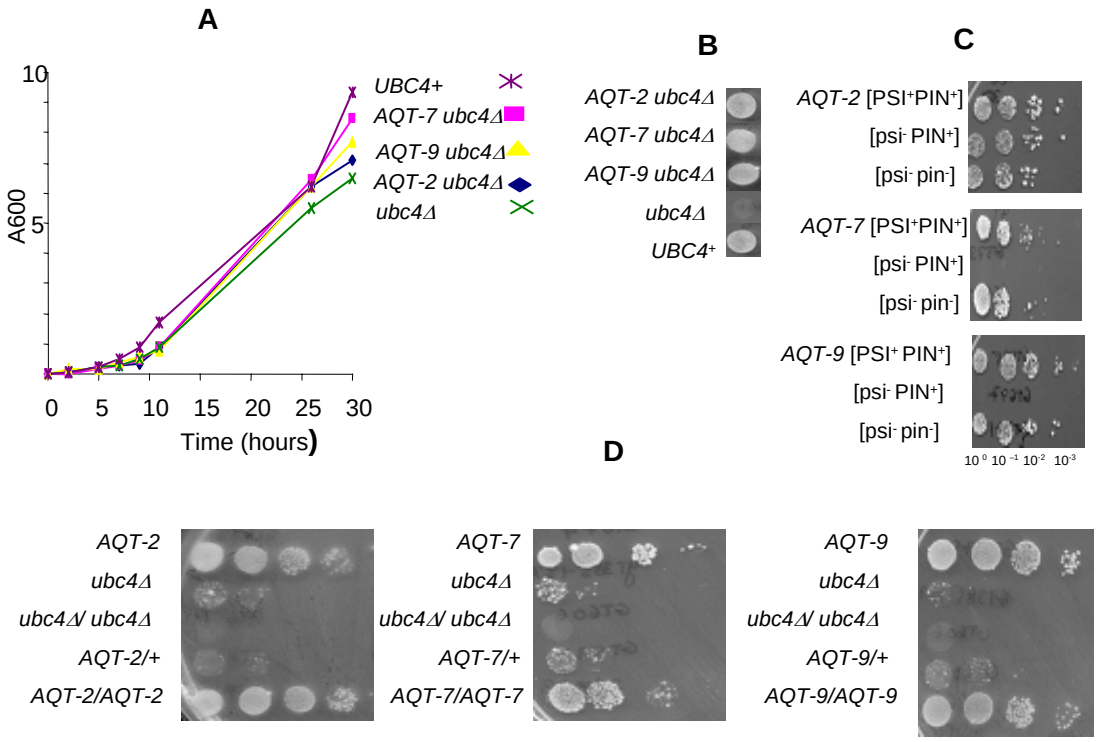


Figure 5.4 Growth characteristics of *AQT* mutants
A. Growth of *AQT* mutants in complete medium. *AQT* [PSI$^+$] mutants along with their controls were grown in YPD medium and their growth was monitored over 30 hrs (see materials and methods - Quantitative analysis for details). B. *AQT* mutants display temperature resistance at 39°C. 10-fold dilutions of equal number of cells (*AQT* [PSI$^+$] mutants and their controls) was spotted onto YPD and synthetic medium SD + 13 (data not shown). YPD plates were photographed after 3 days of incubation at 39°C. As a control, plate made in the same way was kept at 30°C where all strains grew (data not shown). C. *AQT-7* and *AQT-9* [psi$^-$ PIN$^+$] versions show temperature-sensitivity at 39°C. Temperature-sensitivity of all three versions [PSI$^+$ PIN$^+$], [psi$^-$ PIN$^+$] and [psi$^-$ pin$^-$] of *AQT* strain and its isogenic control *ubc4Δ* are compared. 10-fold dilutions of equal number of cells spotted onto 2 YPD plates- the 30°C plate serves as a control for 39°C plate. Plates photographed after 10 days of incubation at 39°C. Control strain remains temperature-sensitive (data not shown). D. Recessiveness of *AQT* by temperature-resistance. The *AQT ubc4Δ/ ubc4Δ* diploids were compared in their temperature-resistance to their haploid parents as well as controls Wild-type *ubc4Δ/ ubc4Δ* diploid and to its homozygous diploid *AQT ubc4Δ/ AQT ubc4Δ*. GT606 (Wild-type *ubc4Δ/ ubc4Δ* diploid), *AQT-2,-7 and -9 ubc4Δ/ ubc4Δ* diploids –GT532,GT534 and GT756 respectively. The homozygous diploid *AQT ubc4Δ/ AQT ubc4Δ- 2,7 and 9* are GT726, GT727 and GT728 (see materials and methods for more details). 10-fold dilutions of equal number of cells were spotted onto YPD and plates were photographed after 4 days of incubation at 39°C. *AQT* [PSI$^+$ PIN$^+$] strains 2, 7, 9 are GT573, GT574 and GT575 respectively along with its isogenic control *ubc4Δ* GT349 and isogenic control *UBC4*$^+$ GT81-1C. *AQT* [psi$^-$ PIN$^+$] strains 2,7,9 are GT675, GT676 and GT677 and *AQT* [psi$^-$ pin$^-$] strains 2, 7 and 9 are GT769, GT770 and GT771. The [psi$^-$ PIN$^+$] and [psi$^-$ pin$^-$] versions of GT349 are GT386 and GT387.

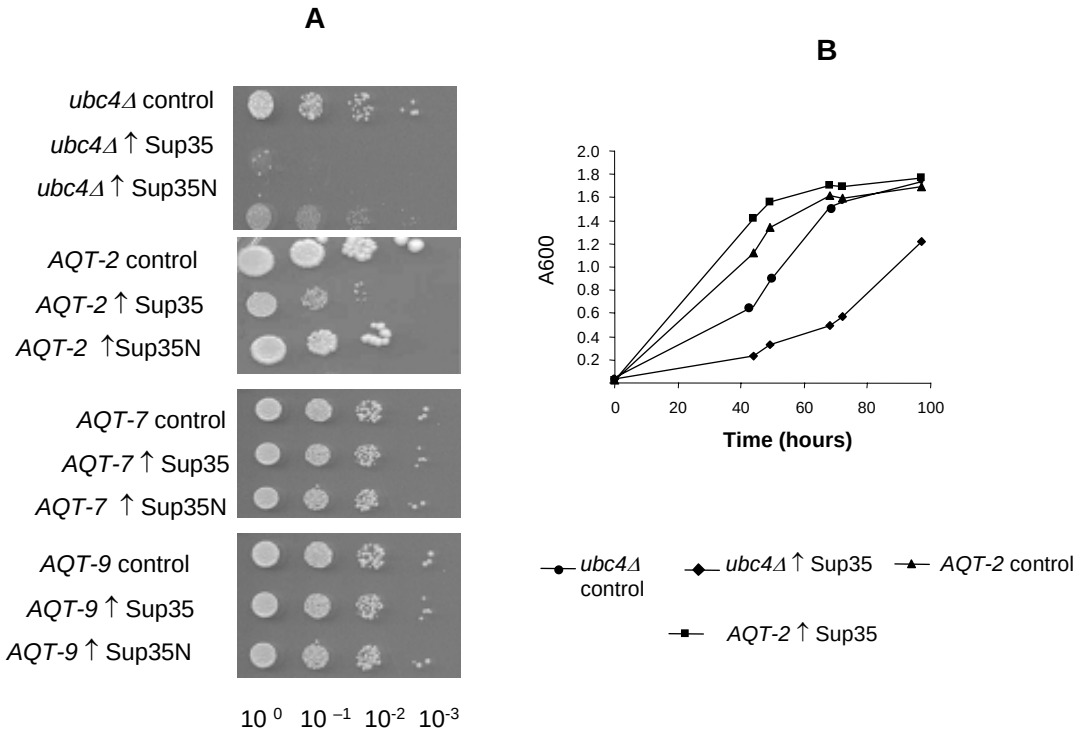


Figure 5.5 *AQT* mutants decrease toxicity of ↑ Sup35/Sup35N
A. Plate Assay. Strain GT349 and the three *AQT* strains *AQT-2* GT573, *AQT-7* GT574 and *AQT-9* GT575 were transformed with plasmids pRS316GAL (control), CEN-GAL-SUP35 (Sup35) and pRS316GALSUP35N (Sup35N), grown on selective medium containing glucose (–Ura/Glu) and equal numbers of cells were then spotted onto selective medium containing galactose and raffinose (-Ura/Gal+Raf) in 10-fold serial dilutions to induce the expression of the Sup35 constructs.Plates were photographed after 10days of incubation at 30°C. B. Quantitative Assay. The same transformants were precultured as given in Materials and Methods. *AQT-7* and *AQT-9* showed similar trend as *AQT-2* (data not shown).

Overproduction of Sup35 increases the probability for the accumulation of misfolded aggregation-prone Sup35 intermediates to adopt the prion form. We investigated if the *AQT* mutants that are able to overcome poly-Q toxicity would do so with another QN rich protein that shares the aggregation-prone property with poly-Q. Indeed we saw that *AQT* mutants can overcome Sup35/Sup35N toxicity with the exception that *AQT-9* strain did not overcome Sup35N toxicity (Figure 5.5 A). Quantitative analysis confirmed our observation (Figure 5.5 B). Data for *AQT-2* strain expressing full length Sup35 is only shown. Trends were similar for *AQT-2* and *AQT-9* expressing Sup35/Sup35N and are not shown. However, when *AQT* mutants were tested with Sup35/Sup35N expressed under its own promoter on a *LEU-2d* based multicopy vector, no such anti-toxicity effect was observed (Figure 5.6 A). The difference in expression levels as indicated before for Cup-huntingtin construct could explain this effect. We also checked the effects of *AQT* mutants on [PSI^+] suppression. *AQT-9* showed better [PSI^+] suppression followed by *AQT-7* than its *ubc4Δ* isogenic control and *AQT-2* (Figure 5.6 B) on –ade medium. *AQT-9* and *AQT-7* continued to show better [PSI^+] suppression when Sup35 was present at high levels ie when checked on –leu-ade medium. Level of [PSI^+] suppression in *AQT-2* was same as its *ubc4Δ* isogenic control in both –ade and –leu-ade medium (Figure 5.6 B).

Effect of *AQT* mutants on poly-Q aggregation and endocytosis

The next logical step was to study the effects on poly-Q aggregation in *AQT* strains. In the yeast system, toxicity of Q103 appears to be directly associated with its aggregation (Meriin et al., 2001). Proposed theories are that aggregates themselves are toxic to cell or that the aggregates could sequester important essential proteins and thus could be cause of toxicity. In this case anti-toxicity could result if the interaction between aggregates and proteins is impaired and the proteins still remain available for function.

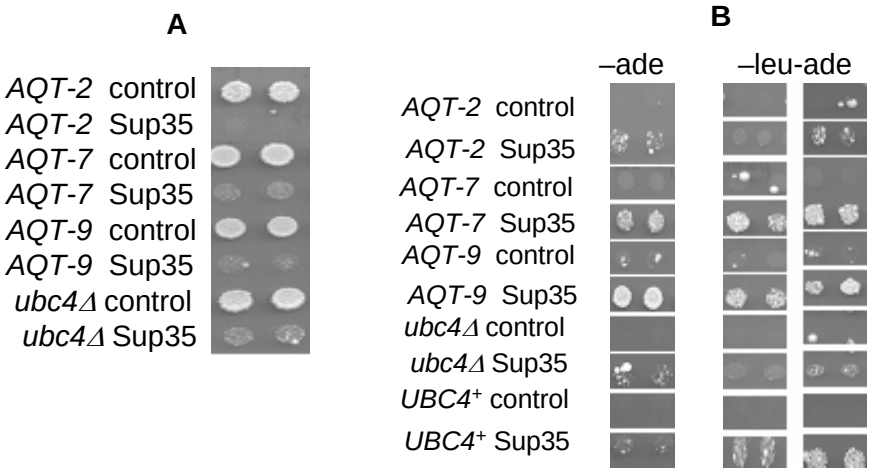


Figure 5.6 Effect of *AQT* mutants on Sup35 toxicity and suppression without the involvement of galactose

A. *AQT* mutants do not counteract Sup35 toxicity. *AQT* [psi$^-$ RNQ$^+$] strains *AQT-2* GT673, *AQT-7* GT674, *AQT-9* GT675 and GT386 –control strain were transformed with pEMBL-YEX (control) and pEMBL-SUP35 (Sup35). Transformants were grown on medium selective for the plasmid containing glucose (-Leu). Images photographed after 15 days of incubation. B. *AQT* [psi$^-$ RNQ$^+$] strains same as mentioned in A. show better [PSI$^+$] suppression over its control. Transformants grown on -Leu were velveteen replica-plated onto –Ade and –Leu-Ade medium. Growth on –Ade medium shows suppression in the presence of moderate copies of *SUP35* and growth on –Leu-Ade medium is an indication of growth with high levels of *SUP35* (See materials and methods for pEMBL constructs). At least 8 independent transformants were tested in each case for each combination. Typical representatives are shown.-Ade plate is photographed after 7 days of incubation and the –Leu-Ade plates photographed after 15 days and 24 days of incubation are shown.

Thus one would expect that in cells showing anti-toxicity effect, the Q103 clumps would be broken down. In case of *AQT* mutants, difference in pattern of aggregation was observed. From ~ 200 cells observed with each mutant, around 60% of *AQT 2* cells expressing Q103 showed dot-like structures while clumps were seen in the rest of them (Figure 5.7 A). 40% of *AQT-7* cells expressing Q103 diffused fluorsescence while clumps were seen in the rest of them. In almost all *AQT-9* cells expressing Q103, clumps were seen, some infact showed even an increased size than its isogenic wild-type controls (Figure 5.7 A). This suggests that atleast in *AQT-2* and *AQT-7* decrease of Q103 aggregates could be a possible mechanism of anti-toxicity. Q103 aggregation in yeast was shown to be associated with defect of endocytosis and has been implicated as one of the likely causes of Q103 toxicity (Meriin et al., 2003). The effect of Q103 aggregates on endocytosis was monitored using lipophilic dye FM4-64 (See materials and methods-chapter 2 Endocytosis section for more details). This dye binds to the cell membrane and is internalized through endocytic pathway. Cells where endocytosis is not affected show a distinct ring around the vacuoles after 45 mins of staining indicating the fusion of dye-stained endosomes with vacuolar membrane. In contrast, all the cells showing Q103 clumps contained no such rings indicating a defect in endocytosis. *AQT-2* and *AQT-7* showed ring like structures inspite of the presence of clumps, while in *AQT-9*, no such rings were observed in the presence of Q103 clumps (Figure 5.7 B). *AQT-2* and *AQT-7* strains do not show a defect in endocytosis in cells with clumps and that could be one of the reasons why this strains show anti-toxicity. While *AQT-9* continues to be defective in endocytosis and the reason of anti-Q103- toxicity in this strain remains unclear.

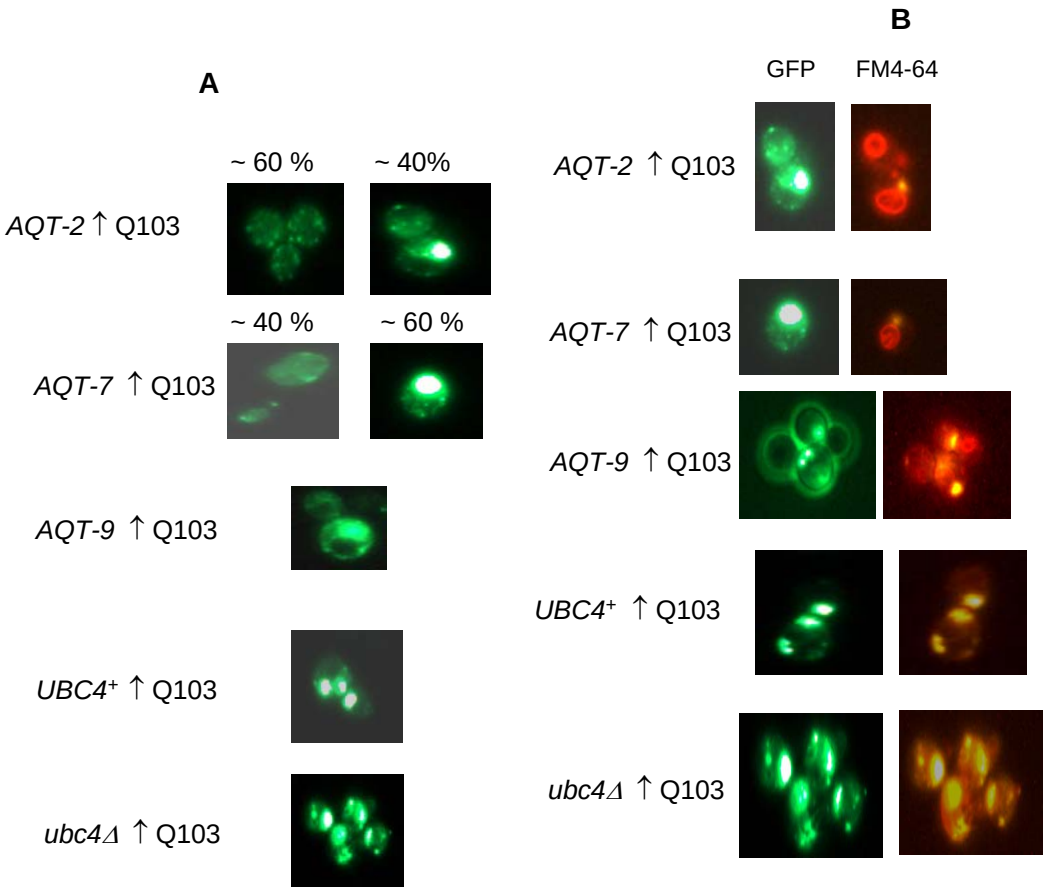


Figure 5.7. Effects on aggregation state of Q103 and endocytosis in *AQT* mutants

A. Types of aggregates in Q103 overexpressing *AQT* strains. Strain GT349 *ubc4Δ*-control for *AQT* strains, three *ubc4Δ AQT* strains *AQT-2* GT573, *AQT-7* GT574 and *AQT-9* GT575 and GT81-1C *UBC4*$^{+}$ strain were transformed with plasmid pYES-103Q. The strains are [PSI^{+} RNQ^{+}]. Transformants were grown in the liquid –Ura medium containing galactose and raffinose (Gal+Raf) to induce the Q103. Distinct types of aggregates were observed with different strains. B. *AQT-2* and *AQT-7* (but not *AQT-9*) compensate for the endocytosis defect caused by Q103. Transformants grown in –Ura/Gal+Raf were checked for endocytosis using FM4-64, a lipophilic dye that enters vacuolar system via endocytic pathway. It stains the vacuolar membrane when endocytosed showing a ring like structure. *AQT-2* and *AQT-7* cells showing Q103 aggregates show ring indicating no defect in endocytosis while *AQT-9* cells with Q103 aggregates do not show a ring, indicating a defect in endocytosis. Both the controls show no rings in cells with Q103 aggregates showing a defect in endocytosis (See Materials and methods –Fluorescence Microscopy Section for details). Note that residual fluorescence of cells with large clumps in the red (FM4-64) filter results from leakage of GFP fluorescence through red filter.

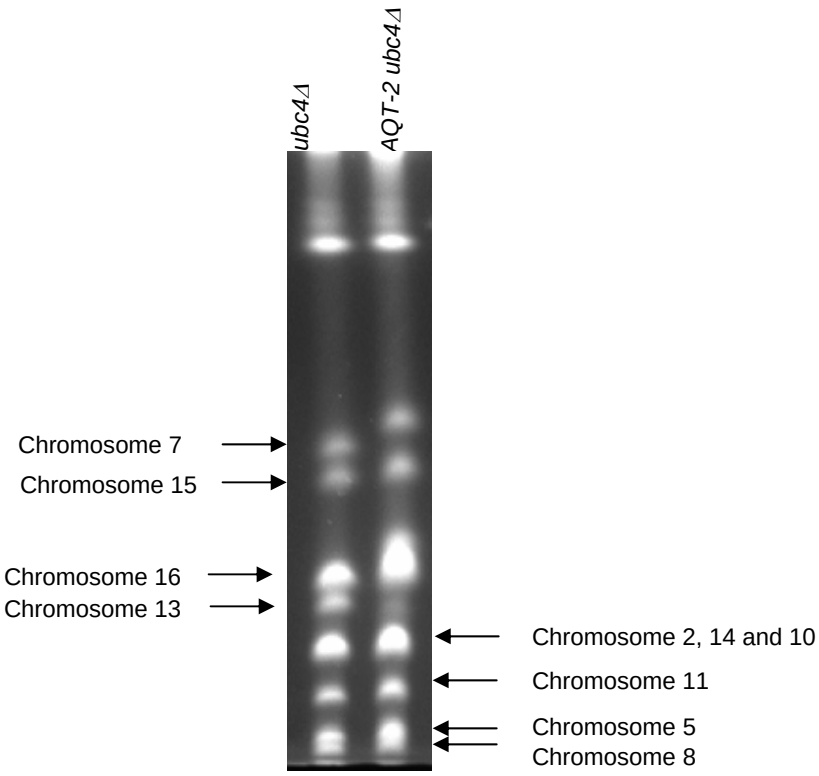


Figure 5.8 Pulse-field gel electrophoresis of chromosomal DNA of *AQT-2* strain GT573 and its control WT GT349 The strains are [PSI$^+$ RNQ$^+$] *ubc4Δ.* The DNA from *AQT-2* strain shows different pattern of bands than its wild-type control – the chromosome 13 band is missing and a intense band at chromosome position 16 could indicate amplification of some parts of chromosome 13 corresponding to a jump in the migration pattern.This data was generously provided by Vidya Narayanan (Dr. Lobachev's laboratory).

Pulse-field gel electrophoresis detected difference in DNA pattern of *AQT* mutant and its *ubc4Δ* isogenic control

Pulse-field gel electrophoresis is used for the seperation of large chromosomal DNA. When the chromosomal DNA from the *AQT-2* strain and its *ubc4Δ* strain were run side by side, it revealed differences in DNA pattern (See figure 5.8). The seperation was good in the range 600 kb-1Mb and it separated out chromosomes 7, 15, 13 and16 very well. The band corresponding to chromosome 13 was missing from *AQT-2* strain's DNA and instead there was a high intensity band at position 16. This indicates a complex rearrangement. The migration pattern of chromosome 7 and 15 was a bit off than its wild-type control but nevertheless, the bands existed. Increase in size of chromosome 7 was also seen with *AQT-7* (data not shown). The band corresponding to chromosomes 2, 14 and 10 migrated as one single band in both the strains. Following was the band corresponding to chromosome 11 followed by a band corresponding to chromosome 5 followed by chromosome 8 (see figure 5.8). All the other chromosome sizes were out of range of seperation of the setup and could not be very well resolved. The identification of chromosomes is preliminary, as our strain chromosome profile is different from chromosomal profile of strains used in Dr. Lobachev's Laboratory.

Discussion

Role of Ubc4 in poly-Q mediated toxicity

Absence of the major Ubiquitin-conjugating enzyme Ubc4 in yeast gave rise to a subset of cells able to overcome poly-Q associated toxicity. Together with Ubc5, Ubc4 is responsible for the majority of ubiquitin-dependent targeting of proteins for degradation via the proteasome. Deletion of Ubc5 however did not have any effect on poly-Q associated toxicity. Infact even excess of Sup35/Sup35N mediated toxicity was lowered

in the *ubc4Δ* background (See Kim D. Allen's thesis for more information). These results implicate that Ubc4 either directly or via interaction or regulation of other proteins favors the poly-Q associated toxicity seen also with another QN-rich protein Sup35. This result actually contradicts the conventional thinking where the absence of Ubc4 would be expected to have an effect on ubiquitination of proteins marked for degradation and will therefore lead to an excess of misfolded proteins causing an increase in toxicity. Yet, another thinking would be that cytotoxicity is due to the efficient recruitment of Ubiquitin-proteosome components (UPS) to protein aggregates in order to effect their elimination. This results in their titration (functional depletion). In concert with this proposal, the efficient targeting of excessive amounts of misfolded proteins and/or aggregates may overwhelm the proteasome, inhibiting its activity. Indeed, impairment of the UPS by protein aggregation has been demonstrated in mammalian cells (Bence ***et al.***, 2001). Such toxicity would be expected to be overcome by: 1) the elimination of protein aggregates; 2) compensation of depletion by overexpression of titrated components; and 3) a decrease in the efficiency of targeting substrates for degradation and/or the recruitment of chaperone and UPS components to targeted substrates. Thus *ubc4*Δ ameliorating toxicity is consistent with this model.

Summarizing the results, the poly-Q antitoxcity phenotype of *AQT* mutants is *ubc4*Δ dependent. The anti-toxicity phenotype of *AQT* mutants seen with both Q103 and Sup35/Sup35N is galactose dependent. Anti-Q103-toxicity phenotype is dominant and is of a mendelian nature. Genetic analysis revealed that the mutations are on the same gene *AQT* mutants show temperature-resistance at 39°C and this temperature-resistance phenotype is recessive. Interestingly, [psi$^-$ PIN$^+$] versions of *AQT-7* and *AQT-9* show some similar trends with repect to Q103 anti-toxicity, temperature-sensitivity at 39°C and levels of [PSI$^+$] suppression. In some fraction of *AQT-2* and *AQT-7* mutants

Q103GFP clumps are not seen and the remainder that show clumps show absence of endocytosis defect. This could possibly be the mechanism for anti-Q103-toxicity. *AQT-9* mutant however continues to show clumps and defect of endocytosis in cells containing clumps and the mechanism of anti-toxicity in this strain remains unclear. Chromosomal DNA of the *AQT-2* strain shows a different pattern than its wild-type *ubc4Δ* control. The absence of band corresponding to chromosome 13 and an increase in intensity of band corresponding to chromosome number 16 could indicate that chromosome 13 has undergone some complex rearrangment likely to be amplification of the portion of chromosome 13 accounting for the increase in size and jump in the migration pattern of DNA.

In the meanwhile, we also engaged in checking some candidate genes most likely to have mutations and which were easily testable. *RNQ1* was tested in a way described in materials and methods. It was tested by a mating approach, and the results stated also in materials and methods indicated that it was not the causative gene. Amongst the other candidates checked were Sla1 and Sla2. The rationale behind checking these proteins was that all three have Q rich regions that could serve as nuclei for Q103 aggregation.

Based on the recessive nature of temperature-sensitive phenotype of *AQT* strains, we tried cloning the gene by library complementation of this phenotype. However, since the temperature-resistance of *AQT* strains was not observable on synthetic medium and due to the mitotic instability of the temperature resistance phenotype, this approach failed. Another approach was to make a library from one of the *AQT* strains and transform the *ubc4Δ* strain to select antitoxic clones. Our pulse-field electrophoresis data indicating complex rearrangment of possibly chromosome 13 and

an increase in size of chromosome 7 obtained in colloboration with Dr Lobachev's laboratory indicates that this approach might not work.

As effects are *ubc4Δ* dependent, increase in copy number of some of the genes on chromosome 13 (if this rearrangement were to be responsible for *AQT* phenotype) that are components of ubiquitination/protein degradation system could be the likely candidates. Indeed such genes like *BUL2*, *CUE4*, *CUE1*, *PRE8*, *PRE5*, *UFO1*, *UBC7*, *ASI1* and *SEL1* are present on chromosome 13. Bul2 is a component of the Rsp5 E3-Ub ligase complex involved in mono-Ubiquitination and poly-Ubiquitination. Cue4 is a protein of unknown function but has a CUE domain that binds to ubiquitin, which may facilitate intramolecular ubiquitination. Cue1 is involved in ubiquitination and degradation at endoplasmic reticulum surface. Pre8 and Pre5 are subunits of 20S proteasome and are involved in Ub-dependent protein catabolism. Ufo1 is a component of Skp1-Cdc53-Fbox receptor (SCF) E3 Ub-ligase that is involved again in Ub-dependent protein catabolism. Ubc7 is an E2 conjugation enzyme, AsiI is a putative integral membrane E3 Ub ligase and Sel1 has an Ub regulatory X domain (UBX) that is responsible for degradation of an ubiquitilated model substrate. A change in expression levels of any of these genes could explain *ubc4Δ* dependent anti-toxicity phenotype in the *AQT* strains. Identification of such a gene whose amplification is responsible for the *AQT* phenotype might need a microarray based approach.

CHAPTER 6

CHIMERIC STUDIES INVOLVING SUP35

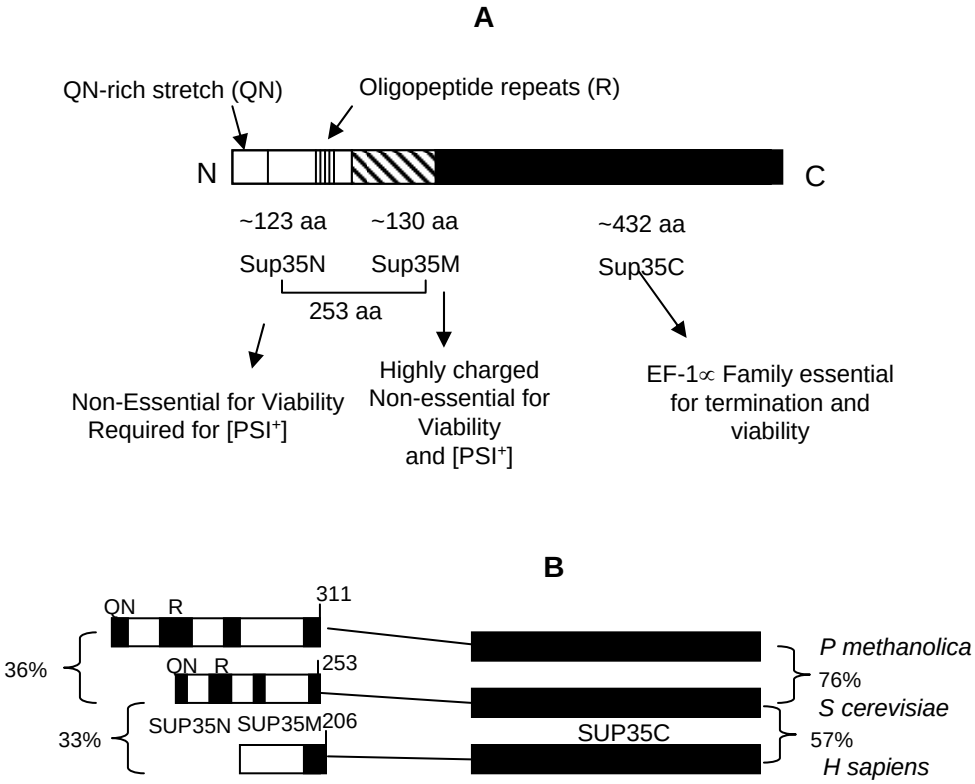
Background

Prion forming potential of Q-rich region of huntingtin

The prion proteins that are otherwise unrelated, share a common feature of prion conversion ie ability to form β-sheet rich amyloid aggregates. In the two best-characterized yeast prions [PSI^+] and [URE3], prion properties depend on glutamine- and asparagine-rich (Q/N rich) domains at the amino terminus of each protein (Serio and Lindquist, 1999; Wickner et al., 1999). The prion domains are dispensable for the normal function of their proteins and are modular, conferring the ability to aggregate when transferred to other proteins (Wickner et al., 2000). Genomic searches for Q/N rich domains similar to Sup35 prion domain led to the identification of prion domains in two uncharacterized proteins, New1p and Rnq1p (Michelitsch and Weissman, 2000; Santoso et al., 2000; Sondheimer and Lindquist, 2000). When Q/N rich regions of these proteins were fused to the C-terminal translation termination domain of Sup35, the resulting fusion proteins could reversibly aggregate to form stable heritable prion states called as [NU^+] and [RNQ^+] which emulated the [PSI^+] nonsense suppression phenotype. Similarly, the mutant form of huntingtin with expanded poly-Q responsible for causing Huntington's disease forms self-seeding amyloid fibrils in vitro (Scherzinger et al., 1999). This commonality inspired us to carry out Huntingtin chimeric Studies and explore if the Q rich regions of Htt when fused to C-terminal domain of Sup35 could emulate the [PSI^+] nonsense suppression phenotype just as seen with New1p and Rnq1p.

Evolution of Sup35p from yeast to humans

Sup35 (erF3) is a well conserved eukaryotic protein. Southern hybridization at low stringency, using *SUP35C* as a probe uncovered *SUP35* homologs from yeast to humans. Infact, *SUP35* from *Pichia methanolica* (Kushnirov et al., 1990), *Homo sapiens* (Jean-Jean, et al., 1996) and *Xenopus laevis* have been cloned and sequenced. Distinct Sup35 domains were seen to exhibit different rates of evolution (Figure 6.1 B). The Sup35C region was conserved from *Saccharomyces* to *Pichia* (76%) and to humans (57%) (Figure 6.1 B). *Pichia SUP35* could complement a deletion of the *S cerevisiae sup35*. In contrast, the Sup35N region is highly variable, even between two yeast genera. They exhibit only 36% homology although *Pichia* Sup35N of 161aa versus *Saccharomyces* Sup35N of 123aa remains QN-rich (Kushnirov, et al., 1990; Santoso, et al., 2000) (see figure 6.1 B, C). The *Pichia* Sup35 PFD-corresponding region is also followed by a portion of the protein, which like S cerevisiae Sup35M, is rich in glutamic acid and lysine residues. The [PSI^+] determinants of *Saccharomyces* Sup35 are the Q/N rich N-terminus (De Pace et al., 1998) and the region of imperfect oligopeptide repeats (Kushnirov et al., 1995; Cox, 1994) that are localized in the 114 N-terminal amino acids of the Sup35 protein (see figure 6.1 A). Although human Sup35N has no significant homology with the corresponding *Saccharomyces* or *Pichia* domains, the first (PFD-corresponding) half of the human Sup35 contains a high percentage of proline (12%) and glycine (26%) residues, which underlies a high probability of turns. Interestingly, the highest similarity was found in the portion corresponding to the *S cerevisiae* Sup35N oligopeptide repeats. However, instead of the regular oligopeptide repeats found in the yeast Sup35 regions, human Sup35 contains long poly-glycine stretches. The second half of the human Sup35N region is enriched in glutamic acid residues (26%) like the *Saccharomyces* and *Pichia* Sup35M. Thus Sup35N domains from different origins show



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	Gly, %	Gln, %	Asn, %
Saccharomyces cerevisiae	17.1	28.5	16.2
Pichia methanolica	11.2	18.7	26.1
Homo sapiens	22.2	2.6	5.1

Figure 6.1 Similarities between Sup35 domains of *Saccharomyces cerevisiae* (yeast used in the lab study), distantly related yeast *Pichia methanolica* and the human *Homo sapiens*
A. Structure of Sup35 revealing important prion determinant features- QN-rich domain and region of imperfect oligopeptide repeats. B. Overall similarity in different domains of Sup35 indicated by percentage between the three species. The C-terminal domain is conserved while the N domain is variable but still shows some homologous regions. Dark regions indicate region of homology and light regions indicate non-homologous regions. C. Amino acid Composition of the Sup35N Regions. Glycine-richness of all three Sup35N domains while *Pichia* and *Saccharomyces* continue to share the Glutamine-Asparagine richness.

high potential of structural flexibility. Chimeric Sup35 ($Sup35^{PS}$) containing the Sup35NM region of *Pichia* fused to MC region of *Saccharomyces* Sup35 could be turned into a prion in *S cerevisiae* by overproduction of identical *Pichia* Sup35NM (Santoso, et al., 2000; Chernoff, et al., 2000; Kushnirov, et al., 2000) as well as complete *P. methanolica* Sup35 protein (Zadorskii, et al., 2000). This chimeric *Pichia-Saccharomyces* prion is designated as $[PSI^{+}]^{PS}$. Prion formation by the chimeric Sup35 proteins containing the Sup35N (or NM) regions from the other yeast genera such as *Candida, Kluyveromyces*, etc. (Santoso, et al., 2000; Nakayashiki, et al., 2001) has also been reported. In each case, prion formation was induced by overproduction of the identical Sup35N (or NM) domain of the same origin, the approach used previously to induce do novo formation of $[PSI^{+}]^{S}$ (Chernoff, et al., 1993; Derkatch, et al., 1996). In this chapter, we explore if N or NM portion of Human Sup35 have such a prion forming potential.

Interspecies prion transfer

Prion state is efficiently transmitted between the cells or organisms of one and the same species via extracellular infection (in mammals) or cytoplasmic exchange (in fungi), the efficiency of interspecies prion transmission is greatly reduced, that is referred to as a “Species Barrier” in prion transmission (Prusiner, 1997; Kocisko, et al., 1995; Hill, et al., 2000). Even evolutionary related mammals (e.g., various species of rodents) exhibit a strict “species barrier”. This barrier could be reproduced in the in vitro conversion experiments, indicating that it is determined by the differences in protein sequences (Kocisko, et al., 1995). However, interspecies prion conversion was observed in certain combinations of mammalian species. For example, PrP^{Sc} transmission from sheep to cattle is believed to be responsible for the BSE epidemics in Europe, while PrP^{Sc}

transmission from cattle is suspected to cause CJD in humans (see Prusiner, 1997). It should be noted that in all cases where interspecies prion infection was detected, the PrP proteins involved were highly homologous to each other and differed only by a few amino acid (aa) positions. Pre-existing $[PSI^+]^S$ prion of *S. cerevisiae* was not able to induce conversion of the Sup35N domain of different origin into a prion state, indicating existence of the strict species barrier in the yeast system (figure 6.2) (Santoso, et al., 2000; Chernoff, et al., 2000; Kushnirov, et al., 2000).

Here, we report that the Saccharomyces-*Pichia* barrier is asymmetric, as pre-existing $[PSI^+]^{PS}$ prion is able to cause conversion of the complete *S. cerevisiae* Sup35 protein ($Sup35^S$) into a prion form, albeit less efficiently.

Materials and Methods

Yeast strains

The *S. cerevisiae* strains used in this work originated from the GT81 series (Chernoff et al., 2000). The haploid segregants that contained the complete deletion of the chromosomal $SUP35^S$ gene (sup35::HIS3) were kept viable with an episomal copy of the endogenous (pASB2: *CEN LEU2 SUP35*) or chimeric *SUP35* gene on the centromeric plasmid as described below. GT393, GT255-2A and GT394 are $[PSI^+]$, $[psi^- PIN^+]$ and $[psi^- pin^-]$ derivatives with deletion of chromosomal copy of Sup35, kept viable with an episomal copy of endogenous Sup35. These strains were used for Human and huntingtin chimeric studies.

Strain GT310 is $[PSI^+]^{PS}$ sup35::HIS3 [*SUP35NMPmCSc*, *URA3*]. GT592 is a $[psi^- PIN^+]$ derivative of GT310 obtained after WT Hsp104 treatment. GT557 is the $[psi^-$

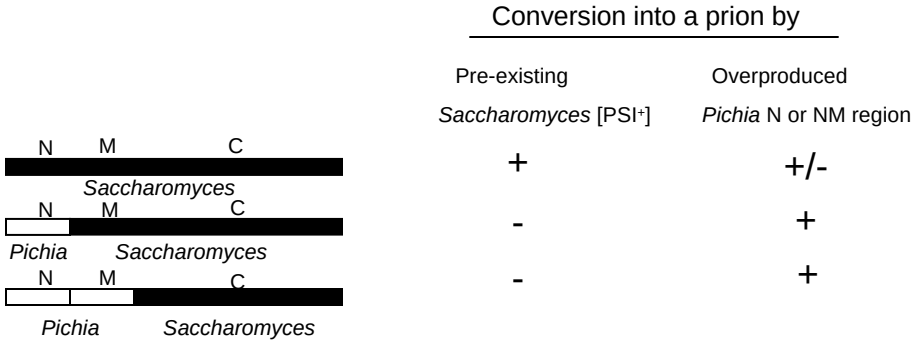

Figure 6.2 Species Barrier
The prion state of *Saccharomyces cerevisiae* endogenous Sup35 cannot be transmitted to next generations via heterologous Sup35p or Sup35N/NM, originating from the distantly related yeast species *Pichia methanolica*

pin$^-$] derivative of GT310 obtained after GuHCl treatment. Strain GT215-8A is *Saccharomyces* [PSI$^+$] Δ sup35::HIS3 [*SUP35Sc*, *URA3*].

GT564 is isogenic to strain GT159 except that the *RNQ1* ORF has been replaced by the *S. pombe HIS5* gene. A 1.4 kb PCR product containing the *S. pombe HIS5* gene flanked by regions of homology to the RNQ1ORF was generated from the plasmid template pFA6a-His3MX6 using the specially designed primers RNQ1FOR (5'CTGAAATGGATACGGATAAGTTAATCTCAGAGGCTGAGTCCGGATCCCCGGGT TAATTAA) and RNQ1REV (5'CAAAGGATAGAAGGCGAACTGAATCATCGTTCAGT AGCGGGAATTCGAGCTCGTTTAAAC) where underlined regions are homologous to regions within and surrounding the *S cerevisiae RNQ1* ORF and non-underlined regions are homologous to the plasmid pFA6a-His3MX6 (Pringle). Linear PCR products generated were transformed into GT159 (psi$^-$ PIN$^+$) strain. Chromosomal DNA from the resulting HIS$^+$ transformants were checked for successful transplacements using the primers RNQ1TESTFOR (5' ATACCCAAGCAAGCTACACG) and RNQ1TESTREV (5' TGAATCATCGTTCAGTAGCGG). Wild-type parental strain was expected to produce 1.31 kb product, while strains carrying the deletion (GT564) produce 1.48 kb band. This strain was transformed with pUCSUP35NMPmCSc and mated with GT310. The haploid segregant from the cross that had deletions in both the genes *SUP35* and *RNQ1* were selected and saved as GT591.

Plasmid constructs

The plasmid pMCup1Q20MCSc was constructed by a two-step strategy. Step1 involved ligating the 260bp PCR-amplified Q20 region from plasmid pAS1HUN20 using forward primers, Hun-ForKav (5' TGGGATCCGCATGGCGAC CCTGGAAAAGCT) and reverse primer, Hun-RevKav (5'TTAGATCTCTCAGCCACAGCCGGGCCGGG) into PCR2.1 cloning vector using enzymes, BamH1 and Bgl II. Primers were designed in a

way so that they were complementary to the beginning and end of the Q20 region and contained the BamH1 and Bgl II sites (underlined) respectively. Step 2 involved excising the BamH1 Bgl II fragment from PCR 2.1 cloning vector and ligating into pMCup1NPmMCSc cut with BamH1. Construction of pMCUp1Q53MCSc was done is a similar way by inserting the 360bp PCR amplified Q53 region of pAS1HUN53 using same set of primers Hun-ForKav and Hun-RevKav into PCR2.1 using BamH1 and Bgl II. Second step is same as second step for pMCup1Q20MCSc. The plasmid pMCUP1Q103McSC was constructed by cloning the BamH1 Bgl II Q103 fragment from pET-Q103 and ligating to pMCup1NPmMCSc cut with BamH1. Exon 1 of huntingtin gene containing 103 Glutamine residues is in frame with MC region of *Saccharomyces* Sup35.

400bp fragment from plasmid p56G (Hoshino et al., 1989) containing the entire human *SUP35* was PCR amplified using using primers Sup35NHs-F (5' AAAGATCTAATCATGGATCCGGGCAGT) and primer, Sup35NHs-R(5' GGGAGATCTGCTAACAGCTGAATTTGA) into PCR cloning vector kit (intermediate step). The primers were designed with Bgl II sites (underlined). This construct was cut with Bgl II and ligated into pMCUP1MSc cut with BamH1. The final construct pMCUP1NHs400MCSc has the N portion of Human *SUP35* fused to MC portion of *Saccharomyces SUP35* in frame was confirmed by sequencing facility of Athens. pYCL-CupNHs400MCSc was constructed by ligating the Xho1 Sac1 fragment of pMCup1NHsMcSc(400) and ligated to pYCL-Cup cut with the same enzymes. This is a *LEU2* based Human chimeric SUP35 construct. Similarly, the NM portion of Human *SUP35* (600bps) was fused to MC portion of *Saccharomyces SUP35* in frame. A 600bp fragment was PCR amplified from plasmid p56G using the existing primers SUP35HsATG-F (5'CCAGATCTATCGATGGATCCGGGCAGTGGC) and SUP35Hs"ATG3"-R (5'GGAGATCTTGGCGGTGCAACCACAGAC). Here again the

primers were designed with Bgl II sites (underlined). The remaining strategy for construction is same as that mentioned for pMCUP1NHs400MCSc. (However this construct could not be completely confirmed as sequencing failed to read sequence beyond 400bp from the primer).

Construction of the chimeric *SUP35*^{PS} gene in which *SUP35NM*^{S} region coding for the first 253 aa was substituted with the first 254 aa of *SUP35NM*^{P} was described previously (Chernoff ***et al.***, 2000). The chimeric *SUP35*^{PS} gene in which only the *SUP35N*^{S} region was substituted with the corresponding *SUP35N*^{P} region was also constructed and used in the shuffle experiments, and produced results similar to those observed in case of the *SUP35NM* substitution. Unless otherwise stated, the designation *SUP35*^{PS} in the figures and table refers to the *SUP35NM*^{P}*-SUP35C*^{S} construct. In all experiments, the *SUP35*^{PS} and *SUP35*^{S} genes were located on the single copy (centromeric) shuttle vectors as described previously (Chernoff ***et al.***, 2000). These genes were placed under the control of the endogenous *SUP35*^{S} promoter. Plasmid pASB2 is a centromeric *LEU2* based vector containing full length *Saccharomyces SUP35* (Borchsenius et al., 2000). Plasmid pYCL-Pm-Sc also a centromeric *LEU2* based vector contains *SUP35NM* region of *Pichia methanolica* fused to *SUP35C* region of *Saccharomyces cerevisiae.*

Results

Can Human chimeric Sup35 be turned into a prion by existing *S cerevisiae* [PSI^{+}]?

The haploid *S. cerevisiae* strains contained complete deletion of chromosomal *SUP35*^{S} gene and possessed both *SUP35*^{HS} and *SUP35*^{S} constructs. *SUP35*^{HS} was expressed under the control of *CUP1 promoter*. When the [*SUP35*^{S}] [PSI^{+}]^{S} strain was transformed with the [*SUP35*^{HS}] plasmid, the resulting transformants lost suppression

only with 600bp of N-terminal sequence of Human Sup35 as described previously (Chernoff, et al., 2000), (Figure 6.3B) and maintained suppression with 400bp of N-terminal sequence of Human Sup35 (figure 6.3 A). In the first case, antisuppression increased with increasing concentration of Copper in the medium, indicating that the effect was caused by Human chimeric Sup35 (Figure 6.3 B). The isogenic [psi$^-$] strains remained Ade$^-$ in either case, confirming that conversion of Sup35^S into a prion state is directed by the pre-existing Sup35^S prion isoform (data not shown). Loss of the [*SUP35^S*] plasmid in such a strain showed suppression (see figure 6.3 C) in case of NHs600MCSc. However, the Ade$^+$ phenotype was not curable by guanidine hydrochloride (GuHCl), agent shown previously to cure [PSI$^+$].

Can glutamine repeats of huntingtin exon 1 substitute for prion forming domain of *S cerevisiae* Sup35 ie can this fusion be turned into a prion?

The haploid *S. cerevisiae* strains contained complete deletion of chromosomal *SUP35^S* gene and possessed both *Q20/Q53/Q103MCSc* and *SUP35^S* constructs. The *Q20/Q53/Q103MCSc* construct was under the control of *CUP1* promoter. In [PSI$^+$] strains, suppression decreased with increasing concentration of CuSO4 and the highest level of antisuppression was seen with Q103MCSc (Figure 6.4 A). The isogenic [psi$^-$ PIN$^+$] strains also showed antisuppression with Q53/Q103MCSc while Q20MCSc showed suppression (Figure 6.4 B). The expectation here was to see the chimeric Sup35 containing Q53 and Q103 domains that are aggregation-prone to increase [PSI$^+$] suppression. On the other hand, they showed antisuppression in [PSI$^+$] cells (figure 6.4 A). This result suggests that these chimeras are non-prion and could efficiently terminate translation. [PSI$^+$] induction by Sup35Sc was also inhibited by Q53 and Q103 chimeras. While on the other hand chimeric Sup35 containing Q20 domain that does not aggregate was expected to should antisuppression, and it infact did not (figure 6.4 A). [PSI$^+$]

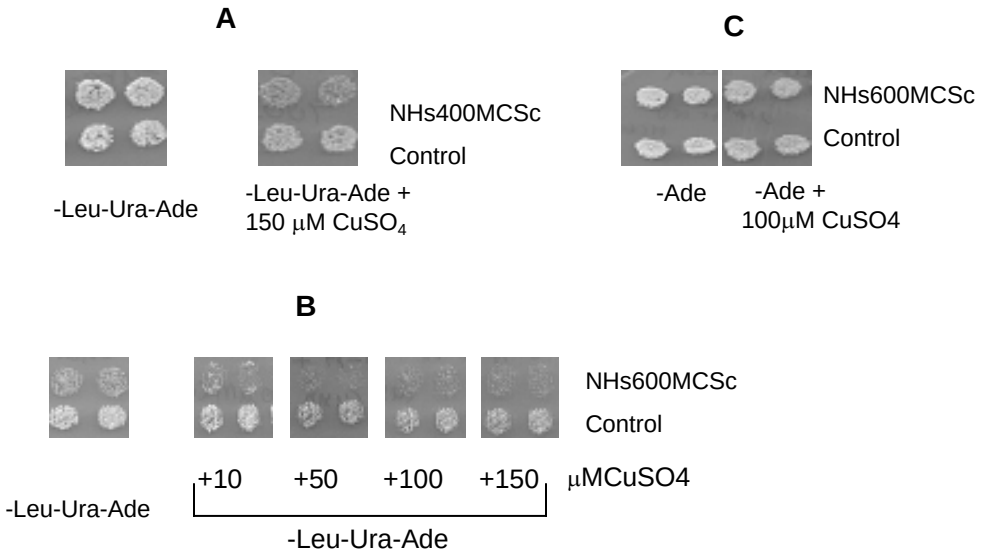

Figure 6.3 Can chimeric Sup35NHsMCSc protein be turned into a prion by pre-existing *S cerevisiae* [PSI$^+$]?
A. NHs400MCSc does not affect suppression by Sup35Sc as seen as growth on selective –ade medium for all concentrations of copper (data not shown for 10, 50, 100 μM CuSO4). Plate is photographed after 7 days of incubation. B. NHs600MCSc shows antisuppression which increases with increasing concentration of CuSO4. Plate is photographed after 5 days of incubation. In both A and B, transformants of the *sup35Δ* containing a combination of *SUP35Sc* (on the *LEU2* plasmid) and chimeric gene (on the *URA3* plasmid) were grown on selective medium –Ura-Leu, velveteened onto selective –ade medium containing different concentration of copper to induce the human chimeric construct. Selective –ade medium without copper serves as a control for non-inducing conditons. The [psi$^-$ PIN$^+$] and [psi$^-$ pin$^-$] transformants with chimeric gene did not exhibit growth on –Ura-Leu-Ade even after 20days (data not shown). C.Yeast containing only Human chimeric Sup35 continue to show suppression but is not prion related. Ura$^+$ Leu$^-$ from [PSI$^+$] NHs600MCSc transformants were selected and checked for suppression on –Ade and -Ade + 10/50/100/150uM CuSO4 (data not shown for all). Plate is photographed after 4 days of incubation. The Ade$^+$ were GuHCl non-curable (data not shown).
Strains used: GT393 [PSI$^+$], GT255-2A [psi$^-$ PIN$^+$] , GT394 [psi$^-$ pin$^-$]. All are *sup35Δ* strains kept alive with the plasmid pASB2 (*CENLEU2SUP35Sc*).
Plasmids used: 'control' – pmCUP; 'NHs400MCSc'-pMCUPNHs400MCSc; 'NHs600MCSc'- pMCUPNHs600MCSc.

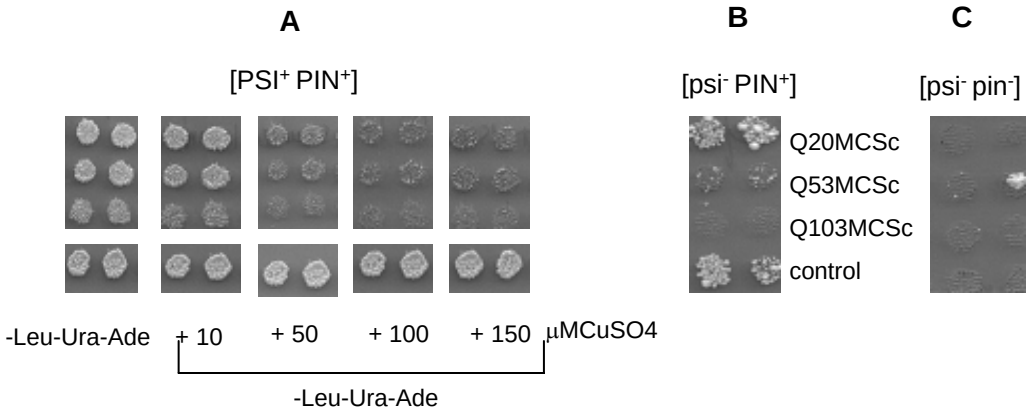

Figure 6.4 Effects of huntingtin chimeric Sup35 on [PSI+] maintenance and induction A. Q20MCSc and Q53MCSc show suppression in the presence of pre-existing [PSI+] while Q103MCSC shows antisuppresion that increases with increasing concentration of CuSO4. B Q20MCSc shows [PSI+] induction while Q53MCSc and Q103MCSc do not support [PSI+] induction C. None of the huntingtin chimeras show suppression in [psi- pin-] strain. Medium selective for both the plasmids Sup35Sc and huntingtin chimeras (-Ura-Leu) is used with different concentration of CuSO4 to induce the huntingtin chimeric constructs.
Strains used are same as that mentioned in Fig 6.3 and plasmids used are ‘Q20MCSc’-pMCUPQ20MCSc; ‘Q53MCSc’-pMCUPQ53MCSc;’Q103MCSc’-pMCUPQ103MCSc

induction by Sup35Sc was not affected by Q20 chimera. None of the chimeric plasmids showed suppression in [psi$^-$ pin$^-$] strain (figure 6.4 C). Strains containing only Huntingtin chimeric constructs could not be obtained. This could be due to the fact that these chimeric constructs were not functional and therefore strain could not be kept alive in the absence of *SUP35*^{S} construct.

[PSI$^+$] induction takes place when *SUP35NM* is fused to *HPR6.6*

Hpr6.6 is a human progesterone receptor protein studied in Dr Choi's laboratory that contains a transmembrane domain. Sup35C also contains a transmembrane domain. It was interesting to check if this transmembrane domain of Sup35C had any role to play in [PSI$^+$] induction and this was tested indirectly by replacing the Sup35C with Hpr6.6 protein forming a chimeric protein Sup35NMHpr6.6

The *HPR* fusion constructs indeed showed [PSI$^+$] induction when expressed under its own promoter and when expressed under the control of *CUP1* promoter ie under both low and high level of expression respectively in [psi$^-$ PIN$^+$] strains of two series (figure 6.5). Induction was the strongest in the strain OT60, while in GT159, it was weak (figure 6.5). [psi$^-$ PIN$^+$] derived from OT56 show induction only *HPR6.6* expressed under its own promoter (figure 6.5 A), while [psi$^-$ PIN$^+$] derived from both OT56 and GT81-1C show induction when *HPR6.6* is expressed under copper inducible promoter (figure 6.5 B).

Asymmetric interspecies prion conversion occurs in the *Pichia-Saccharomyces* system

The presence of the [PSI$^+$]^S or [PSI$^+$]^{PS} prion state, respectively was monitored by growth on –Ade medium (See figure 6.6). When the [*SUP35*^{S}] [PSI$^+$]^S strain was

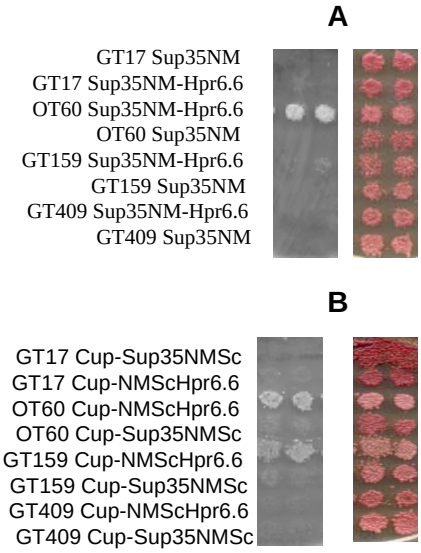

Figure 6.5 Can Sup35NMHPR6.6 chimeric protein expressed from A.*SUP35* promoter and from B. *CUP1* promoter induce [PSI$^+$]? [psi$^-$ PIN$^+$] and [psi$^-$ pin$^-$] derived from OT56 are designated are OT60 and GT17. [psi$^-$ PIN$^+$] and [psi$^-$ pin$^-$] derived from GT81-1C are strains GT159 and GT409 respectively. These strains are transformed with pRS315 based vectors expressing either *SUP35NMSc* or the chimeric *SUP35NMScHPR6.6* under the *SUP35* promoter. The strains were also transformed with pYCL1 based vectors expressing *SUP35NMSc* or the chimeric *SUP35NMScHPR6.6* under the *CUP1* promoter. Transformants were replica velveteen plated onto selective medium to check for suppression (-Leu-Ade) and on YPD to check for [PSI+] by color. Plates were photographed after 7days and 2 days after refrigeration respectively. The transformants expressing *CUP1* constructs were checked onto (-Leu-Ade) and YPD after 3 days of growth on –Leu/Glu + 100uM CuSO4 to induce the constructs. Plate was photographed after 5 days and 2 days after refrigeration respectively. [PSI$^+$] induction was more efficient by chimeric construct in OT60 when expressed from either endogenous or Cu-inducible promoters. [PSI$^+$] induction was favored by GT159 by chimeric construct only when expressed under *CUP1* promoter. [PSI$^+$] induction did not take place in [psi$^-$ pin$^-$] strains regardless of GT or OT background or the promoters.

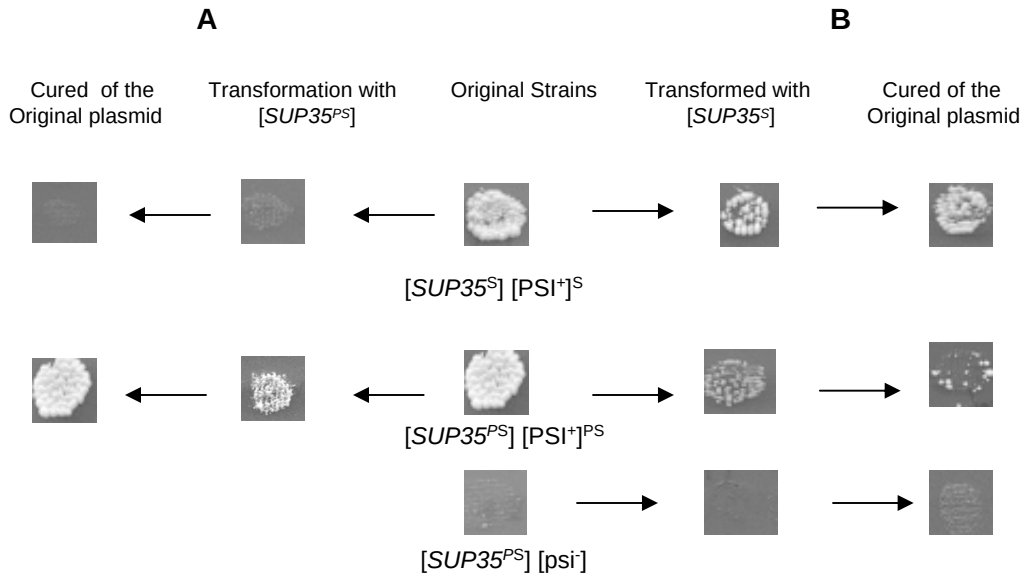

Figure 6.6. Prion conversion in the intraspecies and interspecies shuffle experiments.
Interspecies prion conversion (A) does not occur from *Saccharomyces* prion protein to the chimeric *Pichia-Saccharomyces* protein indicating species barrier (see figure 6.2) (B) but occurs in the opposite direction. Isogenic [RNQ$^+$] strains of *S. cerevisiae* used in this experiment bear the *sup35Δ::HIS3* deletion on the chromosome *and contain the URA3* centromeric plasmids with either the full length *SUP35* gene of *S. cerevisiae* (*SUP35^S*) or the chimeric gene *SUP35^{PS}* (see Chernoff *et al.*, 2000) in which the *SUP35NM* region of *S. cerevisiae* is substituted with the corresponding region of *P. methanolica*. These strains were transformed with plasmids containing *SUP35^S* or *SUP35^{PS}* but with the *LEU2* selective marker. All possible combinations were obtained. Growth of the original strains and transformants on the medium lacking adenine (-Ade) is compared. (To maintain selection for both plasmids, transformants were grown on medium that also lacked uracil and leucine). At the next stage, the original *URA3* plasmids were removed following counterselection against *URA3* marker on the medium with 5-fluoroorotic acid (5-FOA) (see Kaiser *et al.*, 1994). Growth of the clones, resulting from such a plasmid shuffle, on -Ade medium is shown. All plates were incubated for 5 days. Each experiment has been repeated several times with at least 8 independent transformants, with the same results. All strains contain the [RNQ$^+$] prion.All plasmids used in the experiments shown on Fig. 1 contained the *SUP35* genes under the endogenous *S. cerevisiae SUP35* promoter.

transformed with the [*SUP35*^{PS}] plasmid, the resulting transformants lost suppression as described previously (see figure 6.6 A) (Chernoff, et al., 2000), as non-prion Sup35^{PS} protein is sufficient for the efficient termination. Loss of the [*SUP35*^{S}] plasmid in such a strain resulted in the [*SUP35*^{PS}] [psi^{-}] progeny, confirming the existence of the species barrier (Figure 6.6 A). In contrast, the [*SUP35*^{PS}][PSI^{+}]^{PS} strain, transformed with the [*SUP35*^{S}] plasmid, exhibited the heterogenous growth on –Ade medium, indicating that at least a fraction of cells contained the functionally defective (possibly, prion) Sup35^{S} (Figure 6.6 B). After the [*SUP35*^{PS}] plasmid was lost, the resulting [*SUP35*^{S}] progeny were few Ade^{+} papillae, indicating the instability of [PSI^{+}]^{S} and very inefficient conversion (Figure 6.6 B). The isogenic [*SUP35*^{PS}] [psi^{-}] strain remained Ade^{-} (that is, [psi^{-}]) after transformation with [*SUP35*^{S}] and subsequent loss of [*SUP35*^{PS}] (Figure 6.6 B), confirming that conversion of Sup35^{S} into a prion state is directed by the pre-existing Sup35^{PS} prion isoform only when both Sup35^{S} and Sup35^{PS} are present (ie before shuffle).

The interspecies conversion is slower than intraspecies conversion

Transformation of the [*URA3 SUP35*] strains with the [*LEU2 SUP35*] plasmids generated both Leu^{+} Ura^{+} and Leu^{+} Ura^{-} transformants. As Sup35 is essential for viability, the latter class apparently originated from the cells that have lost the “parental” [*URA3*] –marked plasmid right before transformation and still contained a residual Sup35 protein when they were transformed. In case of the Sup35^{PS} intraspecies shuffle, all Leu^{+} Ura^{-} transformants were [PSI^{+}] checked by GuHCl curability, suggesting that even a short-term coexistence of the residual prion with the identical Sup35 protein coded by the newly introduced gene was sufficient to turn the latter protein into a prion. In contrast, almost all Leu^{+}Ura^{-} transformants in the interspecies shuffle ([*URA3 SUP35*^{PS}][PSI^{+}] to [*LEU2 SUP35*^{S}]) remained [psi^{-}] (Figure 6.7 A) in contrast to the Leu^{+}

Ura$^+$ showing only 3-4-fold higher papillatory growth confirmed as [PSI$^+$] by GuHCl curability.

Interspecies prion conversion of the Sup35 protein is independent of the presence or absence of [RNQ$^+$] prion

[RNQ$^+$] is the prion isoform of Rnq1, the QN-rich protein of unknown function (Sondheimer and Lindquist, 2000). [RNQ$^+$] was previously shown to facilitate de novo induction of [PSI$^+$]^S by overproduced Sup35^S (Derkatch et al., 2001, Osherovich and Weissman, 2001) and to increase aggregation (Osherovich and Weissman, 2001, Meriin et al., 2002) and toxicity (Meriin et al., 2002) of the mammalian polyQ proteins in yeast. Induction of [PSI$^+$]^{PS} by overproduced Sup35N^P (Chernoff et al., 2000) was also facilitated in the presence of [PIN$^+$] (Derkatch et al., 1997), later identified with [RNQ$^+$] (Derkatch et al., 2001). We have confirmed that [PIN$^+$] strains of our collection indeed contain the aggregated (that is, prion) form of Rnq1 (Meriin *et al.*, 2002). However, [RNQ$^+$] is not needed for reproduction of pre-existing [PSI$^+$]^S (Derkatch et al., 2001, Derkatch et al., 1997) or [PSI$^+$]^{PS} (Y. Chernoff and E. Lewitin, data not shown). We have observed that both intraspecies shuffles ([*SUP35^S*] [PSI$^+$]^S to [*SUP35^S*] and [*SUP35^{PS}*] [PSI$^+$]^{PS} to [*SUP35^{PS}*]) and interspecies shuffle ([*SUP35^{PS}*] [PSI$^+$]^{PS} to [*SUP35^S*]) produced [PSI$^+$] progeny independently of the presence or absence of [RNQ$^+$] prion (Figure. 6.7 B).

Discussion

The *SUP35^{H400S}* construct did not affect [PSI$^+$] suppression by the endogenous Sup35. indicating that Sup35^{H400S} could adopt a prion form. As after shuffle studies could not be conducted with this particular construct, it could very well mean that the Sup35^{H400S} adopts a non-functinal form and yeast cannot survive in its sole presence.

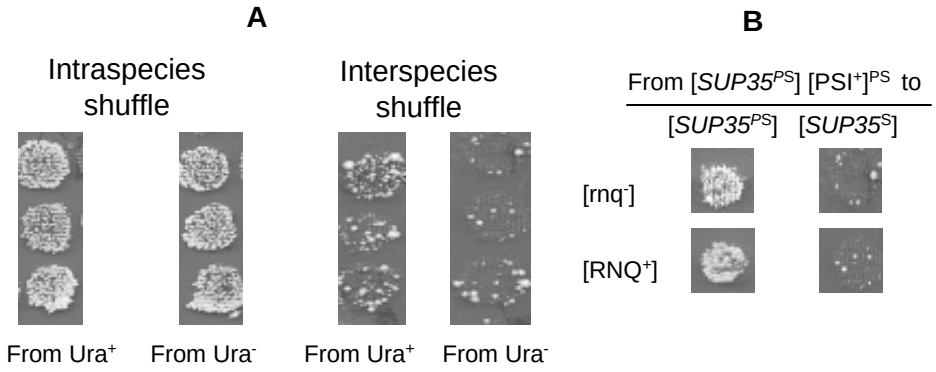

Figure 6.7 Characteristics of the interspecies prion conversion
A. Interspecies conversion requires a long period of co-existence between the inducer and inducee proteins. The [*URA3 SUP35*^{PS}] [PSI^{+}]^{PS} was transformed with the [*LEU2 SUP35*^{PS}] plasmid in intraspecies shuffle and The [*URA3 SUP35*^{PS}] [PSI^{+}]^{PS} was transformed with the [*LEU2 SUP35*^{S}] plasmid in interspecies shuffle . Transformants were obtained either on –Ura-Leu medium selective for both plasmids (left) or on -Leu medium containing 5-fluoroorotic acid (5-FOA) (Kaiser *et al.*, 1994) that counterselects against *URA3* (right). The *URA3* plasmid was eliminated from the former transformants via incubation on 5-FOA after the colonies were formed. [PSI^{+}] is detected by growth on -Ade. Ura^{-} transformants (selected on 5-FOA) remain [psi^{-}]. Intraspecies conversion occurred in both Ura^{+} and Ura^{-} transformants (data not shown and Table 1). 12 independent papillae from each patch of Ura+ (interspecies shuffle) were checked for GuHCl curability and most of them (10/12) remained curable confirming it as [PSI^{+}]. B. Intraspecies and Interspecies conversion is independent of the presence of [RNQ^{+}]. The isogenic [RNQ^{+}] and [rnq^{-}] derivatives of the [*SUP35*^{PS}] [PSI^{+}]^{PS} strain were transformed with either [*SUP35*^{PS}] or [*SUP35*^{S}] plasmids. Plasmid shuffle was performed and presence of [PSI^{+}] detected as growth on –leu-ade. All constructs were under control of the endogenous *SUP35*^{S} promoter.

When yeast contained both *SUP35*^{H600S} and *SUP35*^{S} constructs, suppression is lost. This can be explained by the fact that the non-prion Sup35^{HS} protein is sufficient for the efficient termination. Similar observation was made with *Pichia* chimeric Sup35p (Chernoff et al., 2000). When strain contained only human chimeric Sup35 (NHs600MCSc), the protein remained non-functional, therefore showed growth on –Ade medium. However, the GuHCl test proved this phenomenon to be non-prionic. The fusion protein somehow disrupts the Sup35C portion from terminating translation, independent of it being turned into a prion. Similarly, huntingtin chimeras also remained non-prionic and could efficiently terminate translation. Two possible explanations as to why Hpr6.6 chimeric construct facilitated [PSI^{+}] induction were either due to its destabilization into peptides that would serve as initial nuclei for prion seeding or that Hpr6.6 localises the protein to the membrane increasing the local concentration that in turn form nuclei. There exists a yeast homolog of *HPR6.6* called *YPL170W* that lacks the transmembrane domain and chimeric studies involving this protein would further resolve if it is indeed transmembrane domain facilitating prion induction.

In case of *Pichia* chimeric studies, prion state could be transmitted weakly from the Sup35^{P} prion-forming domain (PFD) to Sup35^{S} PFD (Figure 6.6 B) but not in the opposite direction. This result explains our previous observation (Chernoff, et al., 1995) that overproduction of Sup35N^{P} or complete *Pichia* Sup35 protein (Sup35^{P}) can lead to a weak but detectable [PSI^{+}]^{S} induction in the [psi^{-}] strain containing endogenous Sup35^{S}. Most likely, the overproduced Sup35^{P} or Sup35N^{P} could be turned into the prion isoform, which subsequently converted Sup35^{S} into a prion seen in only fraction of the cells. Asymmetry of interspecies prion conversion in yeast resembles the situation previously observed in mammals, e. g. in the hamster-mouse system (Kocisko *et al.*, 1995). These data are also in agreement with cross-species prion induction observed after overproduction of *Kluyveromyces lactis* Sup35 (Sup35^{KL}) in *S. cerevisiae* (Nakayashiki

et al., 2001). Overproduced $Sup35^{KL}$ was able to induce prion conversion of the endogenous $Sup35^{S}$, but $Sup35^{KL}$ itself could not be induced into a prion by overproduced $Sup35^{S}$. In contrast, the overproduced Sup35 protein from *Yarrovia lipolytica* ($Sup35^{YL}$) was not able to induce $Sup35^{S}$ into a prion, while induction of the $Sup35^{YL}$ prion by overproduced $Sup35^{S}$ was detected (Nakayashiki *et al.*, 2001). However, these experiments, as well as *Pichia-Saccharomyces* cross-induction experiments described in our previous paper (Chernoff *et al.*, 2000), always employed the overexpressor constructs. Therefore, each experiment began with the [psi^{-}] strain, and resulting [PSI^{+}] derivatives apparently originated from a two-step process (intraspecies prion induction followed by the interspecies prion conversion) as described above.

Important distinction of the system presented in the current study is that it operates at the normal protein levels, so that overproduction-dependent de novo prion induction does not make any significant input. This enabled us to specifically characterize the actual step of interspecies prion conversion. We have detected the following important characteristics distinguishing the yeast interspecies conversion from the regular intraspecies conversion. 1) The interspecies conversion is usually less efficient than intraspecies conversion. 2) The interspecies conversion requires a longer period of coexistence of the prion and non-prion proteins in the yeast cell than in case of the intraspecies conversion.

Our data establish the evidence for that the interspecies prion conversion may occur between highly divergent and essentially non-homologous prion-forming domains. This points to the potential danger of the secondary wave of prion transmission once the species barrier is overcome.

CHAPTER 7

EFFECTS OF GLUTAMINE-PROLINE RICH REGIONS OF HUNTINGTIN AND SLA2 ON SUP35/SUP35N TOXICITY AND INDUCTION

Background

Huntingtin and Sla2

Human Htt is a large protein comprising 3144 amino acids. The poly-Q domain, which begins at the 18th amino acid position, usually contains 11-34 glutamine residues in unaffected individuals and expands to > 37 glutamines in HD patients. Two proline-rich domains –(poly-P which contain 11 and 10 prolines) immediately follow the polyQ domain. Proline-rich regions of the protein are implicated to be involved in protein-protein interactions.They form the most flexible region of the sequence. The Q25 and Q103 constructs used previously in chapters 3, 4 and 5 did not contain the proline-rich domains. It was observed that when yeast cells overexpress Q25Pro (N-terminal fragment of Htt with both poly-Q and poly-P domains) in trans with Q103, poly-Q toxicity was eliminated. Similar observation was made when HttPro (N-terminal fragment of Htt containing only the poly-P domain) was expressed in trans with Q103 (M. Sherman, unpublished results). This result suggested that polypeptides that were proline-rich could inhibit aggregation of Q103.

Sup35, another QN-rich protein also shows cytotoxicity when overexpressed in yeast. This protein however does not have any proline rich sequence as seen in the huntingtin protein. In order to understand if proline-rich sequence of Htt were able to counteract aggregation/toxicity of all Q-rich aggregates in general or whether the observation made earlier was only specific with Htt aggregation, we studied its effect on

Sup35/Sup35N toxicity. Overexpression of Sup35/Sup35N induces [PSI$^+$] in cells containing [PIN$^+$], another QN-rich prion proposed to act as nuclei for prion and Htt aggregation. In parallel, we have also studied if proline-rich sequences when expressed in trans influence Sup35/Sup35N induction.

Sla2 like Htt is also rich in glutamine and proline residues. Infact human homolog of Sla2-Hip1 binds to huntingtin (Htt) (Kalchman et al., 1997; Wanker et al., 1997). Sla2 is a member of the widely conserved family of actin-binding proteins. It is essential in yeast for the correct organization of cortical actin cytoskeleton (Holtzman et al., 1993) and is also required for endocytosis (Raths et al., 1993). Sla2 and related proteins all share a similar arrangement of three predicted coiled–coil regions (aa 360-580, aa 700-730, aa 930-960) as well as a C terminal domain similar to the C-terminal domain of talin (see figure 7.1). The proline-rich region is from aa 283-311. The glutamine-rich region is located in the first coiled-coil domain from aa 380-434 (see figure 7.1). In this chapter, we also study Sla2 effects on [PSI$^+$] related toxicity and induction.

Materials and methods

Yeast strains

Yeast strains used in this study are described in Chapter 2.

Plasmid constructs

Plasmids unique to this study are described below. Other commonly used plasmids are described in Chapter 2. Plasmid pYES2-25QP expresses the following protein sequence of the portion of human huntingtin MATLEKLMKAFESLKSFQQQQQQQQQQQQQQQQQQQQQQQ**PPPPPPPPPPP**QLPQPPPQAQPLLPQPQ**PPPPPPPPPP**GPAVAEEPLHRP The first 17amino acid region of huntingtin followed by the poly-Q domain containing 25 Q repeats (underlined section) followed by two proline-rich domains (shown in bold).

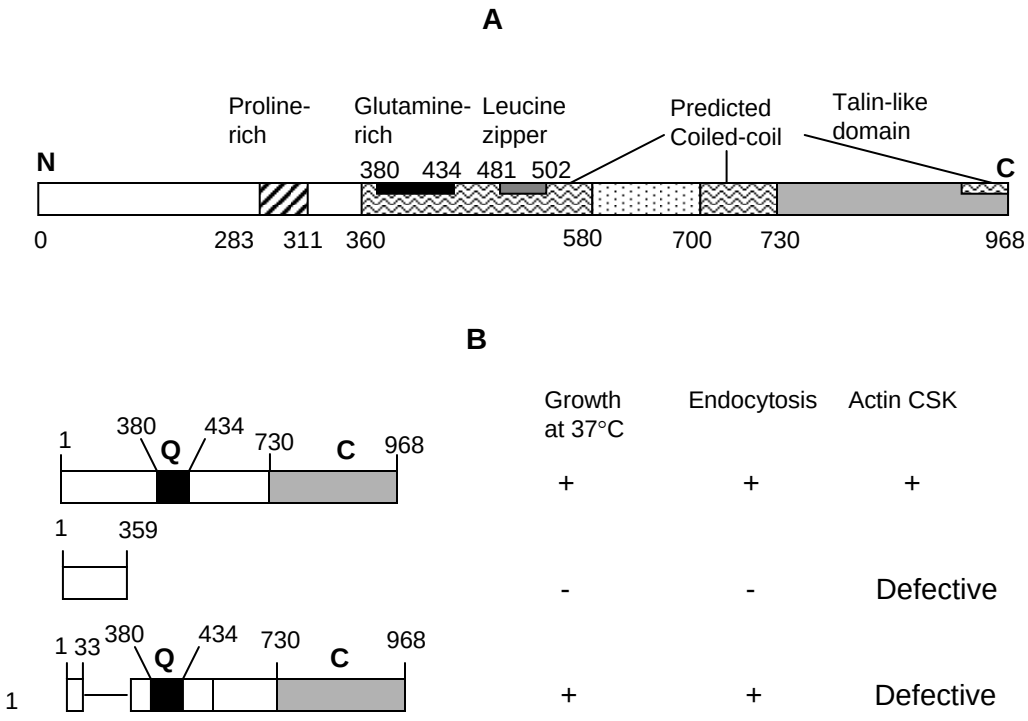

Figure 7.1 Domain arrangement of Sla2
A. Schematic diagram of Sla2. The three predicted coiled-coil forming domains are indicated, as well as glutamine- and proline-rich regions and the C-terminal talin-like domain. B. Deletion Analysis Data (Yang et al., 1999). The *sla2Δ* is temperature-sensitive (ts) and exhibits defects in actin organization and endocytosis.CSK stands for cytoskeleton. The Sla2 fragments retaining Q domain but lacking most of the N-terminal portion (*sla2Δ33-359*) compensates for ts and endocytosis defects but not for actin organization defect. The Sla2 fragment lacking Q domain (*sla2 Δ360-968*) shows *sla2Δ* phenotype.

While plasmid pYES2PRO contains only the region with proline-rich domains following the first 17aminoacids of the huntingtin protein.

Results

Q25Pro effects on Sup35/Sup35N toxicity and Sup35/Sup35N induction

Sup35 is an essential protein functioning in translation termination. In cells containing prions, there is a continuous sequestration of the template protein (Sup35) by prion [PSI^+] aggregates ([PSI^+] is a prion form of Sup35). Such sequestration inhibits growth of strong [PSI^+] variants where amount of soluble Sup35 is already low. Thus Sup35 or even the prion forming domain of Sup35- Sup35N when overexpressed in [PSI^+] cells is toxic. When Q25Pro was expressed along with Sup35/Sup35N, in one of the strong [PSI^+] strains GT81-1C, it inhibited Sup35N toxicity, but did not inhibit toxicity shown by full length Sup35 (Figure 7.2 A). However, in another strong [PSI^+] strain OT56, Q25Pro increased Sup35N toxicity slightly and showed no effect on full length Sup35 (Figure 7.2 A). Excess Sup35N apparently is less toxic in OT56 than in GT81-1C.

Effects of Q25Pro on [PSI^+] induction were checked in the [psi^- PIN^+] derivatives of OT56 and GT81-1C – OT60 and GT159 respectively. Figure 7.2 B shows that Q25Pro increased [PSI^+] induction by Sup35N at a detectable level.

HttPro effects on Sup35/Sup35N toxicity and induction

When HttPro was expressed along with Sup35/Sup35N, it did not modulate Sup35/Sup35N toxicity in GT81-1C (figure 7.3 A). HttPro increased both Sup35/Sup35N in OT56 (figure 7.3 A) as seen with Q25Pro. When checked for [PSI^+] induction, HttPro did not increase [PSI^+] induction to a detectable level as Q25Pro (figure 7.3 B).

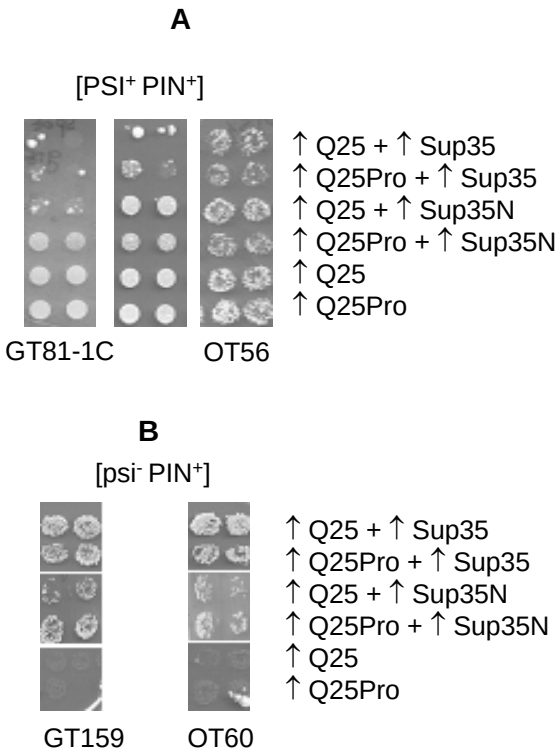

Figure 7.2 Effects of HttQ25Pro on Sup35/Sup35N toxicity and induction
A. Q25Pro decreases Sup35N toxicity in GT81-1C while appears to slightly increase Sup35N toxicity in OT56. [PSI$^+$ PIN$^+$] strains OT56 and GT81-1C were transformed with plasmids mentioned below.Medium used is selective for plasmids and contains galactose to induce the constructs (-Ura-His/Gal).Toxicity plates are photographed after 12 days of incubation. Both spotting and velveteened plates are shown for OT56.
B. HttQ25Pro increases [PSI$^+$] induction by Sup35N [psi$^-$ PIN$^+$] derivatives of OT56 and GT81-1C – OT60 and GT159 respectively were transformed with plasmids mentioned below. Transformants grown on –Ura-His/Gal for 3-5 days were velveteened onto –Ura-His-Ade plates to check for [PSI$^+$] suppression. Plates are photographed after 24 days of incubation. 8 independent transformants were tested and typical representatives are shown.
Plasmids used: ↑ HttQ25: pYES2-25Q; ↑ SUP35: pLA1-SUP35; ↑ SUP35N: pLA1-SUP35N; ↑ HttQ25Pro: pYES2-25QP; Control plasmids: pLA1 and pRS316GAL

Sla2 effects on Sup35/Sup35N toxicity, induction and stability

Excess Sla2 when expressed simultaneously with full length Sup35 facilitates [PSI^+] induction (figure 7.4 A) in [psi^- PIN^+] background. Excess Sla2 ameliorates Sup35 toxicity in weak and strong [PSI^+] -OT55 and OT56 respectively. Sla2 did not have such an effect in GT81-1C another strong [PSI^+] genotypic strain (7.4 B). Excess Sla2 did not affect the stability of [PSI^+] at detectable levels, when checked on YPD for color change after being induced (figure 7.4 C). Yep-based Sla2 facilitated [PSI^+] induction by pEMBL-Sup35, (Figure 7.4 D). Excess Sla2 when expressed from Yep-based vector overcomes pEMBL-Sup35 toxicity only in [psi^- PIN^+] background (Figure 7.4 E). [PSI^+] cells are difficult to be transformed with pEMBL-Sup35 constructs as Sup35 expression levels makes the cells too sick. Thus Sla2 when expressed under gal promoter (Figure 7.4 A and B) and when expressed under its own promoter from a multicopy vector (Figure 7.4 D and E) can overcome Sup35 toxicity and can facilitate [PSI^+] induction.

As overproduction of Sla2 increased [PSI^+] induction by excess Sup35, we checked whether overproduction of Sla2 fragments shown in figure 7.1 B would exhibit the stimulatory effect on [PSI^+] induction. The Sla2 fragment missing the proline-rich domain (sla2Δ33-359) and the full length Sla2 showed similar levels of [PSI^+] induction. But the Sla2 fragment missing the glutamine-rich domain (sla2Δ360-575) showed less [PSI^+] induction than full length Sla2 (see figure 7.5 A). The Sla2 fragments did not modulate the Sla2's ability to show slight anti-toxic effect towards Sup35 (figure 7.5 B).

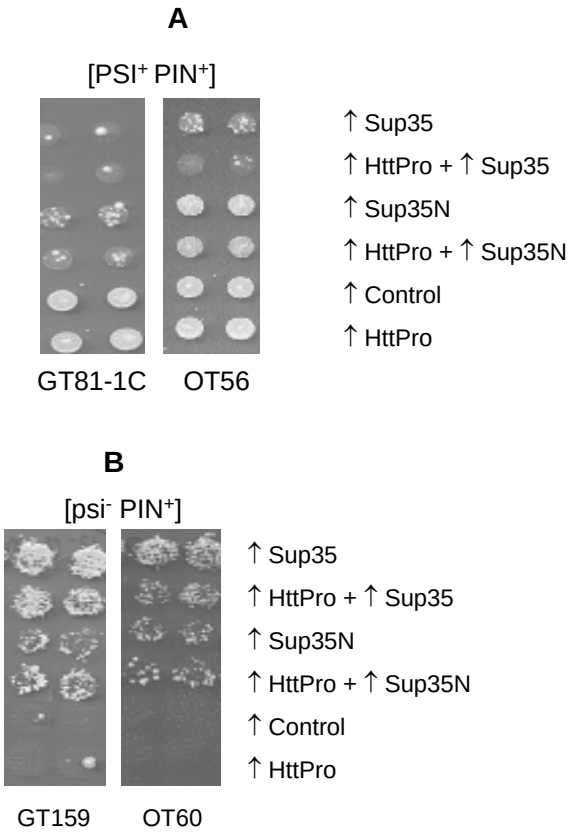

Figure 7.3 Effect of HttPro on Sup35/Sup35N toxicity and induction
A. HttPro increases Sup35/N toxicity in OT56 to a detectable level. [PSI$^+$ PIN$^+$] strains, OT56 and GT81-1C were transformed with plasmids mentioned below.Medium used is selective for plasmids and contains galactose to induce the constructs (-Ura-His/Gal).Toxicity plates are photographed after 26 days of incubation. B. HttPro does not facilitate [PSI$^+$] induction Sup35N at detectable levels. [psi$^-$ PIN$^+$] derivatives of OT56 and GT81-1C – OT60 and GT159 respectively were transformed with plasmids mentioned below. Transformants grown on –Ura-His/Gal for 3-5 days were velveteened onto –Ura-His-Ade plates to check for [PSI$^+$] suppression. Plates are photographed after 24 days of incubation.
Sup35 ,Sup35N and control plasmids used are same as that mentioned in Fig 3.2 legend. ↑ HttPRO: pYES2-PRO.

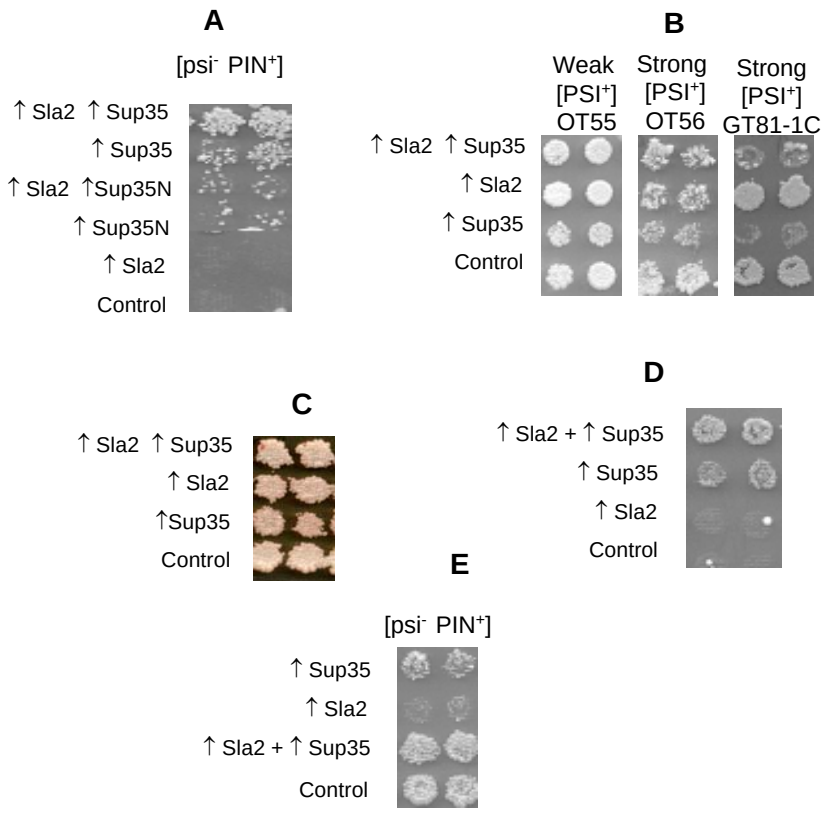

Figure 7.4 Sla2 effects on [PSI+] induction, Sup35 toxicity and stability of [PSI+] A. Excess Sla2 facilitates [PSI+] induction in the [psi- PIN+] strain by only full length Sup35. The transformants are checked for suppression on -Ura-His-Ade/Gal. The plate is photographed after 20 days of incubation. [PSI+] induction does not take place in [psi- pin-] regardless of the strain background and regardless of Sup35/N constructs (data not shown). B. Excess Sla2 overcomes Sup35 toxicity to a detectable level in 74-D694 based [PSI+] strains-OT55 and OT56 but not in GT81 derived GT81-1C. Growth is monitored on –Ura-His/Gal medium and photographed after 10 days of incubation. C. Excess Sla2 does not affect the stability of [PSI+]. -Ura-His and –Ura-His/Gal plates were velveteened onto YPD and curing was checked by appearance of red sectors. A, B and C - Medium used is selective for plasmids and contains galactose to induce Sla2 and Sup35/N constructs. D. 2μ Sla2 facilitates [PSI+] induction by Sup35 as checked by suppression levels on –Ade medium after being velveteened from medium expressing *LEU2-d* series of Sup35 construct. E. 2μ Sla2 constructs overcomes Sup35 toxicity in [psi- PIN+] strain. Medium used is –Ura-His. D and E- Plates are photographed after 10 days of incubation.
The [psi- PIN+] and [psi- pin-] derived from OT56 and GT81-1C: OT60 and GT17 (OT56 derivatives) and GT159 and GT409 (GT81-1C derivatives) respectively.

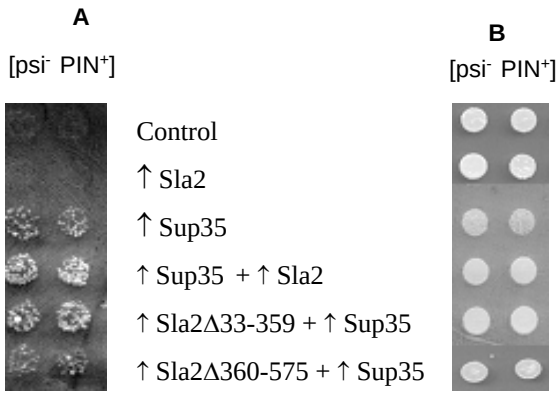

Figure 7.5 Effects of glutamine- and proline- rich regions on A. [PSI+] induction and B. [PSI+] toxicity
A. Glutamine-rich region of Sla2 is required to see [PSI+] induction mediated by Sla2 with full length Sup35. Medium used is selective for plasmids –Ura-His containing galactose to induce the Sup35 construct and lacks adenine to check suppression by [PSI+]. Plate is photographed after 13 days. *Sla2Δ 360-575* lacks glutamine-rich region (see figure 7.1) and suppression is reduced. B. Neither glutamine nor proline rich regions of Sla2 seem to have any effect on the anti-toxic effect of Sla2 on Sup35 toxicity when checked in [psi- PIN+] cells. Just as seen in figure 7.4D full length Sla2 slightly reduces Sup35 toxicity, its truncated versions do not seem to modulate this effect. Medium used was –Ura-His/Gal selective for plasmids and inducing Sup35 construct. Spotting was done to detect slight differences existing if any. Plate was photographed after 11 days. [psi- PIN+] strain: OT60.

Discussion

Proline-rich region of Huntingtin did not overcome Sup35/Sup35N toxicity in contrary to the effects observed with Q103. This implies that anti-Q103-toxicity mechanism of proline-rich sequence of Htt is specific to huntingtin and cannot be extrapolated. Q25Pro infact did overcome Sup35N toxicity implying that glutamine-rich region of huntingtin could interact with glutamine-region of Sup35N domain and possibly reduce toxicity. This however was seen only in one specific genotype GT81-1C strain (Figure 7.2 A). In all other cases both Q25Pro and HttPro increased Sup35N toxicity. The induction and toxicity data fit very well with the OT yeast series (OT56 and OT60), where Q25Pro increases Sup35N toxicity in OT56 [PSI^+ PIN^+] and increases [PSI^+] induction by Sup35N in OT60 [psi^- PIN^+]. This data suggests that *Q25PRO* increases the frequency of the misfolded Sup35N protein to form aggregates thus increasing toxicity in the already prion containing cells.

Excess Sla2 facilitated [PSI^+] induction only in [psi^- PIN^+] strains and not in [psi^- pin^-] strains (data not shown) implicating that Sla2 cannot replace Rnq1's ability to enable prion formation inspite of being glutamine rich like Rnq1. The glutamine-rich region of Sla2 was previously shown to be necessary for growth and endocytosis (Yang et al., 1999) and in this study we also showed it to be important for [PSI^+] induction based on the study with Sla2 fragments.

Thus we conclude that the observation made with Htt with respect to proline-rich sequence did not hold true for Sup35 possibly because Sup35 is not proline-rich itself and therefore does not facilitate any interaction. Yet quite interestingly, glutamine-rich sequence seems to reduce Sup35 toxicity as seen with Q25Pro on Sup35N toxicity in GT81-1C and Sla2 on Sup35 toxicity. Glutamine-rich region of Sla2 and Htt also facilitate [PSI+] induction. Sla1, another member of cytoskeleton associated with actin

patches also assisted [PSI^{+}] formation and propagation (Bailleul et al., 1999). It is possible that actin patches play an important role in [PSI^{+}] induction rather than in [PSI^{+}] propagation as ribbon like structures associated with actin patches are seen in [psi^{-} PIN^{+}] cells where [PSI^{+}] is induced. Sla1 and Sla2 possess structural features similar to prion forming domains eg: Sla1 contains oligopeptide repeats (Holtzman et al., 1993) and Sla2 contains a QN-rich region (Michelitsch and Weissman, 2000). Though, neither of them were found to form prions themselves, their prion forming domains could act as intial “seed” for nucleation of prion polymerization just as seen with actin polymerization.

CONCLUSIONS

1. Dominant negative Hsp104 mutants antagonized poly-Q toxicity and aggregation by eliminating endogenous yeast prions.

2. Some mutant derivatives of Hsp104 with alterations in the middle or C-terminal domain, Hsp104-A503V and Hsp104-C*, ameliorated poly-Q toxicity and decreased size of poly-Q aggregates without affecting prion propagation.

3. Hsp104-A503V increased size of the Sup35 prion aggregates, providing the first known case of synthetic lethality between the chaperone mutation and a prion.

4. Kinetics of [PSI^+] curing by the dominant negative Hsp104 derivative (Hsp104-KT) was shown to be different from [PSI^+] curing by the Hsp104-inactivating chemical GuHCl.

5. Among the yeast Hsp70 chaperones, only Ssa4 was capable of counteracting poly-Q toxicity.

6. Different members of the Hsp40 family, Sis1 and Ydj1, were shown to exhibit opposite effects on poly-Q toxicity. Ydj1 increased prion-dependent poly-Q aggregation and toxicity, while Sis1 decreased size of poly-Q aggregates and antagonized poly-Q toxicity.

7. Interaction between *Saccharomyces* Sup35 and Hsp70-Ssa proteins was detected by two-hybrid assay.

8. Anti poly-Q toxicity mutants called *AQT 2, 7 and 9* were shown to be *ubc4Δ* dependent and temperature-resistant at 39°C.

9. Interspecies prion conversion in *Pichia-Saccharomyces* system is shown to be asymmetric.

10. Huntingtin fragment containing poly-Q and Pro-rich region was shown to facilitate de novo [PSI^+] induction by excess Sup35N.

11. Excess of Sla2, an actin assembly protein with Q-rich region, was shown to increase de novo [PSI^+] induction by excess Sup35 and decrease toxic effect of excess Sup35, confirming role of cytoskeletal networks in de novo prion formation and toxicity.

REFERENCES

1. Allen, K.D., R.D. Wegrzyn, T.A. Chernova, S. Müller, G.P. Newnam, P.A. Winslett, K.B. Wittich, K.D. Wilkinson and Y.O. Chernoff (2004) Hsp70 chaperones as modulators of prion life cycle: novel effects of Ssa and Ssb on the Saccharomyces cerevisiae prion [PSI^{+}]. Genetics Nov. 15 [Epub ahead of print].

2. Avila, J. (2000) Tau aggregation into fibrillar polymers: taupathies**.** FEBS Lett. 2000 476, 89-92.

3. Bailleul, P.A., G.P. Newnam, J.N. Steenbergen and Y.O. Chernoff (1999) Genetic study of interactions between the cytoskeletal assembly protein sla1 and prion-forming domain of the release factor Sup35 (eRF3) in Saccharomyces cerevisiae. Genetics 153, 81-94.

4. Bailleul-Winslett, P.A., G.P. Newnam, R.D. Wegrzyn and Y.O. Chernoff (2000) An anti-prion effect of the anticytoskeletal drug latrunculin A in yeast. Gene Expr. 9, 145-156.

5. Barnett, M.E., A. Zolkiewska and M. Zolkiewski (2000) Structure and activity of ClpB from *Escherichia coli* Role of the amino- and carboxyl-terminal domains. J. Biol. Chem. 275, 37565-37571.

6. Bence, N.F., R.M. Sampat and R.R. Kopito (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292,1552-1555.

7. Bessen, R.A., D.A. Kocisko, G.J. Raymond, S. Nandan, P.T. Lansbury and B. Caughey (1995) Non-genetic propagation of strain-specific properties of scrapie prion protein. Nature 375, 698-700.

8. Bonneaud, N.O., Ozeir-Kalogeropoulos, G.Y. Li, M. Labouesse, M. Minvielle-Sebastia and F. Lacroute (1991) A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/ E coli* shuttle vectors. Yeast 7, 609-615.

9. Borchsenius, A.S., A.A. Tchourikova and S.G. Inge-Vechtomov (2000) Recessive mutations in SUP35 and SUP45 genes coding for translation release factors affect chromosome stability in Saccharomyces cerevisiae. Curr. Genet. 37, 285-291.

10. Bradley, M.E., H.K. Edskes, J.Y. Hong, R.B. Wickner and S.W. Liebman (2002) Interactions among prions and prion "strains" in yeast. PNAS 99, 16392-16399.

11. Broach, J.R., J.N. Strathern and J.B. Hicks (1979) Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene 8, 121-133.

12. Bukau, B. and A.L. Horwich (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92, 351-366.

13. Cao, F., J.J. Levine, S.H. Li and X.J. Li (2001) Nuclear aggregation of huntingtin is not prevented by deletion of chaperone Hsp104. Biochim. Biophys. Acta. 1537, 158-166.

14. Cashikar, A.G., E.C. Schirmer, D.A. Hattendorf, J.R. Glover, M.S. Ramakrishnan, D.M. Ware, and S.L. Lindquist (2002) Defining a pathway of communication from the C-terminal peptide binding domain to the N-terminal ATPase domain in a AAA protein. Mol. Cell. 9, 751-760.

15. Cattaneo, E., D. Rigamonti, D. Goffredo, C. Zuccato, C., F. Squitieri and S. Sipione (2001) Loss of normal huntingtin function: new developments in Huntington's disease research. Trends Neurosci. 24, 182-188.

16. Cesareni, G. and A.H. Murray (1987) Plasmid vectors containing the replication origin of filamentous single-stranded phages. In: Genetic Engineering: Principles and Methods Volume 4. J.K. Setlow (ed.) Plenum Press, New York.

17. Chai, Y., S.L. Koppenhafer, S.J. Shoesmith, M.K. Perez and H.L. Paulson (1999) Evidence for proteasome involvement in polyglutamine disease: localization to nuclear inclusions in SCA3/MJD and suppression of polyglutamine aggregation in vitro. Hum. Mol. Genet. 4, 673-682.

18. Chan, H.Y., J.M. Warrick, G.L. Gray-Board, H.L. Paulson and N.M. Bonini (2000) Mechanisms of chaperone suppression of polyglutamine disease: selectivity, synergy and modulation of protein solubility in Drosophila. Hum. Mol. Genet. 9, 2811-2820.

19. Chacinska, A., B. Szczesniak, N.V. Kochneva-Pervukhova, V.V. Kushnirov, M.D. Ter-Avanesyan and M. Boguta (2001) Ssb1 chaperone is a [PSI^{+}] prion-curing factor. Curr. Genet. 39, 62-67.

20. Chernoff, Y.O., M.V. Ptyushkina, M.G. Samsonova, G.I. Sizonencko, Y.I. Pavlov, M.D. Ter-Avanesyan and S.G. Inge-Vechtomov (1992) Conservative system for dosage-dependent modulation of translational fidelity in eukaryotes. Biochimie 74, 455-461

21. Chernoff, Y. O., S.G. Inge-Vechtomov, I.L. Derkach, M.V. Ptyushkina, O.V. Tarunina, A.R. Dagkesamanskaya and M.D. Ter-Avanesyan (1992) Dosage-dependent translational suppression in yeast Saccharomyces cerevisiae. Yeast 7, 489-499.

22. Chernoff, Y.O., I.L. Derkach and S.G. Inge-Vechtomov (1993) Multicopy *SUP35* gene *induces de-novo* appearance of *psi*-like factors in the yeast *Saccharomyces cerevisiae*. Curr. Genet. 24, 268-270.

23. Chernoff, Y.O., S.L. Lindquist, B. Ono, S.G. Inge-Vechtomov and S.W. Liebman (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. Science 268, 880-884.

24. Chernoff, Y. O., G. P. Newnam, J. Kumar, K. Allen and A. D. Zink (1999) Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone Ssb in formation, stability, and toxicity of the [PSI] prion. Mol. Cell. Biol. 19, 8103-8112.

25. Chernoff, Y. O., A. P. Galkin, E. Lewitin, T. A. Chernova, G. P. Newnam, and S. M. Belenkiy (2000) Evolutionary conservation of prion-forming abilities of the yeast Sup35 protein. Mol. Microbiol. 35, 865-876.

26. Chernoff, Y.O. (2001) Mutation processes at the protein level: is Lamarck back? Mutat. Res. 488, 39-64.

27. Chernoff, Y.O., S.M. Uptain and S.L. Lindquist (2002) Analysis of prion factors in yeast. Methods Enzymol. 351, 499-538.

28. Chernoff, Y. O. (2004). Replication vehicles of protein-based inheritance. Trends Biotechnol. 22, 549-552.

29. Chernova, T. A., K.D. Allen, L.M. Wesoloski, J.R. Shanks, Y.O. Chernoff and K.D. Wilkinson (2003) Pleiotropic effects of Ubp6 loss on drug sensitivities and yeast prion are due to depletion of the free ubiquitin pool. J. Biol. Chem. 278, 52102-52115.

30. Chien, P. and J.S. Weissman (2001) Conformational diversity in a yeast prion dictates its seeding specificity. Nature 410, 223-227.

31. Ciechanover, A. (2003) The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. Biochem Soc Trans. 31, 474-481.

32. Cox, B.S. (1965) PSI, a cytoplasmic suppressor of super-suppressor in yeast. Heredity 20, 505-521.

33. Cox, B. (1994) Cytoplasmic inheritance. Prion-like factors in yeast. Curr. Biol. 4, 744-748.

34. Cummings, C.J., M.A. Mancini, B. Antalffy, D.B. DeFranco, H.T. Orr, and H.Y. Zoghbi (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. Nat. Genet. 2,148-154.

35. Cummings, C.J., Y. Sun, P. Opal, B. Antalffy, R. Mestril, H.T. Orr, W.H. Dillmann and H.Y. Zoghbi (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. Hum. Mol. Genet. 10, 1511-1518.

36. Cyr, D.M. and M.G. Douglas (1994) Differential regulation of Hsp70 subfamilies by the eukaryotic DnaJ homologue YDJ1.J. Biol Chem. 269, 9798-9804.

37. Davies, S. W., M. Turmaine, B.A. Cozens, M. DiFiglia, A.H. Sharp, C.A. Ross, E. Scherzinger, E.E. Wanker, L. Mangiarini and G. P. Bates (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90, 537-548.

38. DebBurman, S.K., G.J. Raymond, B. Caughey and S. Lindquist (1997) Chaperone-supervised conversion of prion protein to its protease-resistant form. Proc Natl Acad Sci U S A. 94, 13938-13943.

39. Derkatch, I. L., Y.O. Chernoff, V.V Kushnirov, S.G. Inge-Vechtomov and S.W. Liebman (1996) Genesis and variability of [PSI] prion factors in Saccharomyces cerevisiae. Genetics 144, 1375-1386.

40. Derkatch, I. L. M.E. Bradley, P. Zhou, Y.O. Chernoff and S.W. Liebman (1997) Genetic and environmental factors affecting the de novo appearance of the [PSI+] prion in Saccharomyces cerevisiae. Genetics 147, 507-519.

41. Derkatch, I. L., M. E. Bradley, S. V. Masse, S. P. Zadorsky, G. V. Polozkov, S. G. Inge-Vechtomov, and S. W. Liebman (2000) Dependence and independence of [PSI+] and [PIN+]: a two-prion system in yeast? EMBO J. 19, 1942-1952.

42. Derkatch, I.L., M.E. Bradley, J.Y. Hong and S.W. Liebman (2001) Prions affect the appearance of other prions: the story of [*PIN*$^+$]. Cell 106, 171-182.

43. DePace, A.H., A. Santoso, P. Hillner and J.S. Weissman (1998) A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. Cell 93, 1241-1252.

44. Dickinson,A.G., and V.M. Merkle (1971) Host-genotype and agent effects in scrapie incubation: change in allelic interaction with different strains of agent. *Mol. Gen. Genet.*, 112, 73-79.

45. Eaglestone, S.S., L.W. Ruddock, B.S. Cox and M.F. Tuite (2000) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [*PSI*$^+$] of Saccharomyces cerevisiae. PNAS USA. 97, 240-244.

46. Faber, P.W., J.R. Alter, M.E. MacDonald and A.C. Hart (1999) Polyglutamine-mediated dysfunction and apoptotic death of a Caenorhabditis elegans sensory neuron. PNAS USA. 96, 179-184.

47. Fernandez-Bellot, E., E. Guillernet and C. Cullin (2000) The yeast prion [URE3] can be greatly induced by a functional mutated *URE2* allele. Embo J. 19, 3215-3222.

48. Ferreira, P.C., F. Ness, S.R. Edwards, B.S. Cox and M.F. Tuite (2001) The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation. Mol Microbiol. 40, 1357-1369.

49. Finley, D. (2002) Ubiquitin chained and crosslinked. Nat. Cell Biol. 4, E121-E123.

50. Glover, J.R. and S. Lindquist (1998) Hsp104, Hsp70 and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell 94, 1-20.

51. Gokhale, K.C., G.P. Newnam, M.Y. Sherman and Y.O. Chernoff (2005) Modulation of prion-dependent poly-Q aggregation and toxicity by chaperone proteins in the yeast model. J. Biol. Chem. Manuscript submitted

52. Goloubinoff, P., A. Mogk, A.P. Zvi, T. Tomoyasu and B. Bukau (1999) Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc Natl Acad Sci U S A. 96, 13732-13737.

53. Hardy J., K. Duff, K.G. Hardy, J. Perez-Tur and M.Hutton (1998) Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. Nat. Neurosci. 1, 355-358.

54. Hartl, F. U. (1996) Molecular chaperones in cellular protein folding. Nature 381, 571-579.

55. Heller, J., A.C. Kolbert, R. Larsen, M. Ernst, T. Bekker, M. Baldwin, S.B. Prusiner, A. Pines and D.E. Wemmer (1996) Solid-state NMR studies of the prion protein H1 fragment. Protein Sci. 5, 1655-1661.

56. Hershko, A., and A. Ciechanover (1998) The ubiquitin system. Annu. Rev. Biochem. 67,425-479.

57. Hill, A.F., S. Joiner, J. Linehan, M. Desbruslais, P.L. Lantos and J. Collinge (2000) Species-barrier-independent prion replication in apparently resistant species. PNAS USA 97, 10248-10253.

58. Holtzman, D.A., S. Yang and D.G. Drubin (1993) Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. J. Cell. Biol. 122, 635-644.

59. The Huntington's Disease Collaborative Research Group (1993). A novel gene containing a trinuleotide repeat that is unstable on Huntington's disease chromosomes. Cell 72, 971-973.

60. Jackson, G.R., I. Salecker, X. Dong, X. Yao, N. Arnheim, P.W. Faber, M.E. MacDonald and S.L. Zipursky (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of Drosophila photoreceptor neurons. Neuron 3, 633-42.

61. James, P., C. Pfund and E.A. Craig (1997) Functional specificity among Hsp70 molecular chaperones. Science 275**,** 387-389.

62. Jean-Jean, O., X.L. Goff and M. Philippe (1996) Is there a human [psi]? C. R. Acad. Sci. III. 319, 487-492

63. Jung, G., G. Jones, R.D. Wegrzyn and D.C. Masison (2000) A role for cytosolic hsp70 in yeast [PSI$^+$] prion propagation and [PSI$^+$] as a cellular stress. Genetics 156, 559-570.

64. Jung, G., G. Jones and D.C. Masison (2002) Amino acid residue 184 of yeast Hsp104 chaperone is critical for prion-curing by guanidine, prion propagation, and thermotolerance. PNAS USA 99, 9936-9941.

65. Jones, G.W. and D.C. Masison (2003) Saccharomyces cerevisiae Hsp70 Mutations Affect [*PSI*$^+$] Prion Propagation and Cell Growth Differently and Implicate Hsp40 and Tetratricopeptide Repeat Cochaperones in Impairment of [*PSI*$^+$]. Genetics 163, 495-506.

66. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163-168.

67. Kaiser, C., S. Michaelis, and A. Mitchell (1994) Methods in yeast genetics: A Cold Spring Harbor Laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

68. Kalchman, M.A., H.B. Koide, K. McCutcheon, R.K. Graham, K. Nichol, K. Nishiyama,P. Kazemi-Esfarjani, F.C. Lynn, C. Wellington, M. Metzler, Y.P. Goldberg, I. Kanazawa, R.D. Gietz and M.R. Hayden (1997) HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. Nat. Genet. 16, 44-53.

69. Kazemi-Esfarjani, P and S. Benzer (2000) Genetic suppression of polyglutamine toxicity in Drosophila. Science 287, 1837-1840.

70. Kazantsev, A., E. Preisinger, A. Dranovsky, D. Goldgaber and D. Housman (1999) Insoluble detergent-resistant aggregates form between pathological and nonpathological lengths of polyglutamine in mammalian cells. PNAS USA. 96, 11404-11409.

71. Kimura, Y., I. Yahara and S. Lindquis (1995) Role of the protein chaperone YDJ1 in establishing Hsp90-mediated signal transduction pathways. Science 268,1362-1365.

72. Kimura, Y., S. Koitabashi, A. Kakizuka and T. Fujita (2001) Initial process of polyglutamine aggregate formation in vivo. Genes Cells. 6, 887-897.

73. Kocisko, D.A., S.A. Priola, G.J. Raymond, B. Chesebro, P.T. Lansbury Jr and B. Caughey (1995) Species specificity in the cell-free conversion of prion protein to protease-resistant forms: a model for the scrapie species barrier. PNAS USA 92, 3923-3927.

74. Krobitsch, S. and S. Lindquist (2000) Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. PNAS USA 97, 1589-1594.

75. Kurtz, S., J. Rossi, L. Petko, and S. Lindquist (1986) An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. Science 231, 1154-1157.

76. Kushnirov, V.V., M.D. Ter-Avanesyan, M.V. Telckov, A.P. Surguchov, V.N. Smirnov and S.G. Inge-Vechtomov (1988) Nucleotide sequence of the SUP2 (SUP35) gene of Saccharomyces cerevisiae. Gene 66, 45-54.

77. Kushnirov, V.V., M.D. Ter-Avanesian, V.N. Smirnov, I. Chernov, I.L. Derkach, O.N. Novikova , S.G. Inge-Vechtomov, M.A. Neistat and Tolstorukov II (1990) [Comparative analysis of the structure of SUP2 genes in Pichia pinus and Saccharomyces cerevisiae] Mol. Biol. (Mosk). 24, 1024-1036.

78. Kushnirov, V.V., M.D. Ter-Avanesian and V.N. Smirnov (1995) [Structure and functional similarity of yeast Sup35p and Ure2p proteins to mammalian prions] Mol. Biol. (Mosk). 29, 750-755.

79. Kushnirov, V.V., D.S. Kryndushkin, M. Boguta, V.N. Smirnov and M D. Ter-Avanesyan (2000) Chaperones that cure yeast artificial [*PSI*$^{+}$] and their prion-specific effects. Curr. Biol. 10, 1443-1446.

80. Lansbury, P.T. and B. Caughey (1995) The chemistry of scrapie reaction: the “ice 9” metaphore. Chem. Biol. 2, 1-5.

81. Li, S. and X. Li (2004) Huntingtin-protein interactions and the pathogenesis of Huntington’s disease. Trends Genet. 20, 146-154.

82. Liu, H., J. Krizek and A. Bretscher (1992) Construction of a GAL1-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics 132, 665-673.

83. Liu, J.J., and S. Lindquist (1999) Oligopeptide-repeat expansions modulate ‘protein-only’ inheritance in yeast. Nature 400, 573-576.

84. Mangiarini, L., K. Sathasivam, M. Seller, B. Cozens, A. Harper, C. Hetherington, M. Lawton, Y. Trottier, H. Lehrach, S.W. Davies and G.P. (1996) Exon 1 of the *HD* Gene with an Expanded CAG Repeat Is Sufficient to Cause a Progressive Neurological Phenotype in Transgenic Mice. Cell 87, 493-506.

85. Martindale, D., A. Hackam, A. Wieczorek, L. Ellerby, C. Wellington, K. McCutcheon, R. Singaraja, P. Kazemi-Esfarjani, R. Devon, S.U. Kim et al. (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. Nat. Genet. 18, 150-154.

86. Mayer, R.J (2003) From neurodegeneration to neurohomeostasis: the role of ubiquitin. Drug News Perspect. 16, 103-108.

87. Meriin, A.B., X. Zhang, X. He, G.P. Newnam, Y.O. Chernoff and M.Y. Sherman (2002) Huntingtin toxicity in yeast model depends on polyglutamine aggregation mediated by a prion-like protein Rnq1. J. Cell. Biol. 157, 997-1004.

88. Meriin, A.B., X.Zhang, N.B. Miliaras, A. Kazantsev, Y.O. Chernoff, J.M. McCaffery, B. Wendland and M.Y. Sherman (2003) Aggregation of Expanded Polyglutamine Domain in Yeast Leads to Defects in Endocytosis. Mol. Cell. Biol. 23, 7554-7565.

89. Michelitsch, M.D. and J.S. Weissman (2000) A census of glutamine/asparagines-rich regions: Implications for their conserved function and the prediction of novel prions. PNAS USA 22, 11910-11915.

90. Moriyama, H., H.K. Edskes and R.B. Wickner (2000) [URE3] prion propagation in *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. Mol. Cell Biol. 20, 8916-22.

91. Muchowski, P.J., G. Schaffar, A. Sittler, E.E. Wanker, M.K. Hayer-Hartl and F.U. Hartl (2000) Hsp70 and Hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. PNAS USA. 97, 7841-7846.

92. Muchowski, P.J., K. Ning, C. D'Souza-Schorey and S. Fields (2002) Requirement of an intact microtubule cytoskeleton for aggregation and inclusion body formation by a mutant huntingtin fragment. PNAS USA. 99, 727-732.

93. Nakayashiki,T., K. Ebihara, H. Bannai and Y. Nakamura (2001) Yeast [PSI^+] "prions" that are crosstransmissible and susceptible beyond a species barrier through a quasi-prion state. Mol. Cell. 7, 1121-1130.

94. Nelson, R.J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne and E.A. Craig (1992) The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. Cell 71, 97-105.

95. Ness, F., P. Ferreira, B.S. Cox and M.F. Tuite (2002) Guanidine hydrochloride inhibits the generation of prion "seeds" but not prion protein aggregation in yeast. Mol. Cell Biol. 22, 5593-55605.

96. Neuwald, A.F. and L. Arvind, J.L Spouge and E.V. Koonin (1994) AAA^+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. 9, 27-43.

97. Newnam, G.P., R.D. Wegrzyn, S.L. Lindquist and Y.O. Chernoff **(**1999) Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. Mol Cell Biol. 19, 1325-1333.

98. Nguyen, J., M.A. Baldwin, F.E. Cohen and S.B. Prusiner (1995) Prion protein peptides induce alpha-helix to beta-sheet conformational transitions. Biochemistry. 34, 4186-4192.

99. Nieto-Sotelo, J., K.B. Kannan, L.M. Martinez and C. Segal (1999) Characterization of a maize heat-shock protein 101 gene, HSP101, encoding a ClpB/Hsp100 protein homologue. Gene 230, 187-195.

100. Nieto-Sotelo, J., L.M. Martinez, G. Ponce, G.I. Cassab, A. Alagon, R.B. Meeley, J.M. Ribaut, R. Yang (2002) Maize HSP101 plays important roles in both

induced and basal thermotolerance and primary root growth. 14, 1621-1633.

101. Nucifora, F.C., M. Sasaki, M.F. Peters, H. Huang, J.K. Cooper, M. Yamada, H. Takahashi, S. Tsuji, J. Troncoso, V.L. Dawson et al. (2001) Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science 291, 2423-2428.

102. Ogura, T., and A.J. Wilkinson (2001) AAA^+ superfamily ATPases: common structure-diverse function. Genes Cells 6, 575-597.

103. Osherovich, L. Z. and J.S. Weissman (2001) Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion. Cell 106, 183-194.

104. Osherovich, L. Z., Cox, B. S., Tuite, M.F., and Weissman, J. S. (2004) *PLoS Biol.* **2**,1-10

105. Patino, M.M., J.J. Liu, J.R. Glover and S. Lindquist (1996) Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science 273, 622-626.

106. Parsell, D. A., Y. Sanchez, J. D. Stitzel, and S. Lindquist. (1991) Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. Nature 353, 270-273.

107. Parsell, D.A., A.S. Kowal, M.A. Singer and S. Lindquist. (1994) Protein disaggregation mediated by heat-shock protein Hsp104. Nature 372, 475-478.

108. Paulson, H.L., M.K. Perez, Y. Trottier, J.Q. Trojanowski, S.H. Subramony, S.S. Das, P. Vig, J.L. Mandel, K.H. Fischbeck and R.N. Pittman (1997) Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 19, 333-344.

109. Paushkin S.V., V.V. Kushnirov, V.N. Smirnov and M.D. Ter-Avanesyan (1996) Propagation of the yeast prion-like [psi^+] determinant is mediated by oligomerization of the *SUP35*-encoded polypeptide chain release factor. *EMBO J.* 15, 3127-3134.

110. Perez, M.K., H.L. Paulson, S.J. Pendse, S.J. Saionz, N.M. Bonini and R.N. Pittman (1998) Recruitment and the role of nuclear localization in polyglutamine-mediated aggregation. J. Cell Biol. 143, 1457-1470.

111. Perutz, M.F., T. Johnson, M. Suzuki and J.T. Finch (1994) Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. PNAS USA 91, 5355-5358.

112. Perutz M.F. (1999) Glutamine repeats and neurodegenerative diseases: molecular aspects. Trends Biochem. Sci. 24, 58-63.

113. Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. Science 216, 136-144.

114. Prusiner,S.B. (1997) Prion diseases and the BSE crisis. Science 278, 245-251.

115. Prusiner, S.B., and M.R. Scott (1997) Genetics of prions. Annu. Rev. Genet. 31, 139-175.

116. Prusiner, S.B., M.R. Scott, S.J. DeArmond and F.E. Cohen (1998) Prion protein biology Cell 93, 337-348.

117. Qian, X., W. Hou, L. Zhengang and B. Sha (2002) Direct interactions between molecular chaperones heat-shock protein (Hsp) 70 and Hsp40: yeast Hsp70 Ssa1 binds the extreme C-terminal region of yeast Hsp40 Sis1. Biochem. J. 361, 27-34.

118. Queitsch, C., S.W. Hong, E. Vierling and S. Lindquist (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. Plant Cell 12, 479-492.

119. Raths, S., J. Rohrer, F. Crausaz and H. Riezman (1993) end3 and end4: two mutants defective in receptor-mediated and fluid-phase endocytosis in Saccharomyces cerevisiae. J. Cell Biol. 120, 55-65.

120. Roberts, B.T., Moriyama, H., and Wickner, R.B. (2004) [URE3] prion propagation is abolished by a mutation of the primary cytosolic Hsp70 of budding yeast. Yeast 21, 107-117.

121. Ross, C.A. and C.M. Pickart (2004) The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases. Trends Cell Biol. 14, 703-711.

122. Rubinsztein, D. C., Leggo, J., Coles, R., Almqvist, E., Biancalana, V., Cassiman, J. J., Chotai, K., Connarty, M., Crauford, D., Curtis, A., Curtis, D., Davidson, M. J., Differ, A. M., Dode, C., Dodge, A., Frontali, M., Ranen, N. G., Stine, O. C., Sherr, M., Abbott, M. H., Franz, M. L., Graham, C. A., Harper, P. S., Hedreen, J. C., Hayden, M. R., et al. (1996). Phenotypic characterization of individuals with 30-40 CAG repeats in the Huntington disease (HD) gene reveals HD cases with 36 repeats and apparently normal elderly individuals with 36-39 repeats. Am J Hum Genet. 59,16-22.

123. Sakahira, H., P. Breuer, M.K. Hayer-Hartl and F.U. Hartl (2002) Molecular chaperones as modulators of polyglutamine protein aggregation and toxicity. PNAS USA 99, 16412-16418.

124. Sambrook, J. and D.W. Russel (2001) Molecular Cloning: A laboratory Manual (3rd Editin). Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

125. Sanchez , Y. and S.L. Lindquist (1990) HSP104 required for induced thermotolerance. Science 248, 1112-1115.

126. Sanchez, Y., J. Taulien, K.A. Borkovich, and S.L. Lindquist. (1992) Hsp104 is required for tolerance to many forms of stress. EMBO J. 11, 2357-2364.

127. Sanchez, Y., D.A. Parsell, J. Taulien, J.L. Vogel, E. A. Craig and S. Lindquist (1993) Genetic evidence for a functional relationship between Hsp104 and Hsp70. J. Bacteriol. 175, 6484-6491.

128. Santoso, A., P. Chien, L.Z. Osherovich and J.S. Weissman (2000) Molecular basis of a yeast prion species barrier, Cell 100, 277-288.

129. Satyal, S.H., E. Schmidt, K. Kitagawa, N. Sondheimer, S. Lindquist, J.M. Kramer and R.I. Morimoto (2000) Polyglutamine aggregates alter protein folding homeostasis in *Caenorhabditis elegans*. PNAS USA 97, 5750-5755.

130. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H. and Wanker, E. E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates *in vitro* and *in vivo*. Cell 90, 549-558.

131. Scherzinger, E., A. Sittler, K. Schweiger, V. Heiser, R. Lurz, R. Hasenbank, G.P. Bates, H. Lehrach and E.E Wanker (1999) Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. PNAS USA 96, 4604-4609.

132. Schirmer, E.C, S. Lindquist and E. Vierling (1994) An Arabidopsis heat shock protein complements a thermotolerance defect in yeast. Plant Cell 6, 1889-1909

133. Schirmer, E.C., J.R. Glover, M.A. Singer and S. Lindquist (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. 21, 289-296.

134. Schirmer, E.C., C. Queitsch, A.S. Kowal, D.A. Parsell and S. Lindquist (1998) The ATPase activity of Hsp104, effects of environmental conditions and mutations. J. Biol. Chem. 273, 15546-15552.

135. Schirmer, E. C., O.R. Homann, A.S. Kowal and S. Lindquist (2004) Dominant gain-of-function mutations in Hsp104p reveal crucial roles for the middle region. Mol. Biol. Cell 15, 2061-2072.

136. Schwirmer, C. and D.C. Masison (2002) Antagonistic interactions between yeast [PSI^+] and [URE3] prions and curing of [URE3] by Hsp70 protein chaperone Ssa1p but not by Ssa2p. Mol. Cell. Biol. 22, 3590-3598.

137. Serio, T.R., and S.L. Lindquist (1999) [PSI+]: an epigenetic modulator of translation termination efficiency. Annu. Rev. Cell Dev. Biol. 15, 661-703.

138. Serio, T.R., and S.L. Lindquist (2000) Protein-only inheritance in yeast: something to get [PSI+]-ched about. Trends Cell Biol. 10, 98-105.

139. Sherman, F. (2002). Getting started with yeast. Methods Enzymol. 350**,** 3-41.

140. Sherman, M.Y., and A.L. Goldberg (2001) Cellular defenses against unfolded proteins: a cell biologist thinks about neurodengenerative diseases. Neuron 29, 15-32.

141. Shorter, J. and S. Lindquist (2004) Hsp104 catalyzes formation and elimination of self-replicating Sup35 prion conformers. Science 304, 1793-1797.

142. Sikorski, R.S., and P. Hieter (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122, 19-27.

143. Smith, C.K., T.A. Baker and R.T. Sauer (1999) Lon and Clp family proteases and chaperones share homologous substrate-recognition domains. PNAS USA 96, 6678-6682.

144. Sondheimer, N. and S. Lindquist (2000) Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell 5**,** 163-172.

145. Sondheimer, N., N. Lopez, E.A. Craig and S. Lindquist (2001) The role of Sis1 in the maintenance of the [RNQ+] prion. Embo J. 20, 2435-2442.

146. Ter-Avanesyan, M. D., V.V. Kushnirov, A.R. Dagkesamanskaya, S.A. Didichenko, Y.O. Chernoff, S.G. Inge-Vechtomov and V.N. Smirnov (1993) Deletion analysis of the SUP35 gene of the yeast Saccharomyces cerevisiae reveals two non-overlapping functional regions in the encoded protein. Mol Microbiol. 7, 683-692.

147. Ter-Avanesyan, M.D., A.R. Dagkesamanskaya, V.V. Kushnirov and V.N. Smirnov (1994) The SUP35 omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [psi+] in the yeast Saccharomyces cerevisiae. Genetics 137: 671-676.

148. Tran, P.B. and R.J. Miller (1999) Aggregates in neurodegenerative disease: crowds and power? Trends Neurosci. 22, 194-197.

149. Trojanowski, J.Q., M. Goedert, T. Iwatsubo, and V.M.Lee (1998) Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia. Cell Death Differ. 10, 832-837.

150. Trojanowski, J.Q. and V.M. (2000) "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. Ann. N. Y. Acad. Sci. 924, 62-67.

151. Uptain, S.M., G.J. Sawicki, B. Caughey and S.L. Lindquist (2001) Strains of [*PSI*$^{+}$] are distinguished by their efficiencies of prion-mediated conformational conversion. EMBO J. 20, 6236-6245.

152. Vale, R.D. (2000) AAA proteins. Lords of the ring. J. Cell Biol. 150, F13-F19.

153. Vieira, J., and J. Messing (1991) New pUC-derived cloning vectors with different selectable markers and DNA replication origins Gene 100, 189-194.

154. Tuite, M.F., C.R. Mundy and B.S. Cox (1981) Agents that cause a high frequency of genetic change from [psi+] to [psi-] in Saccharomyces cerevisiae. Genetics 98, 691-711.

155. Waelter, S., A. Boeddrich, R. Lurz, E. Scherzinger, G. Lueder, H. Lehrach and E.E. Wanker (2001) Accumulation of mutant huntingtin fragments in aggresome-like inclusion bodies as a result of insufficient protein degradation. Mol. Biol. Cell 5, 1393-1407.

156. Wanker, E.E., C. Rovira, E. Scherzinger, R. Hasenbank, S. Walter, D. Tait, J. Colicelli and H. Lehrach (1997) HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum. Mol. Genet. 6, 487-95.

157. Warrick, J.M., H.Y. Chan, G.L. Gray-Board, Y. Chai, H.L. Paulson and N.M. Bonini (1999) Suppression of polyglutamine-mediated neurodegeneration in Drosophila by the molecular chaperone HSP70. Nat Genet. 23, 425-428.

158. Wegrzyn, R.D., K. Bapat, G.P. Newnam, A.D. Zink and Y.O. Chernoff (2001) Mechanism of prion loss after Hsp104 inactivation in yeast. Mol. Cell. Biol. 21, 4656-4669.

159. Werner-Washburne, M., and E.A. Craig (1989) Expression of members of the Saccharomyces cerevisiae hsp70 multigene family. Genome 31, 684-689.

160. Wickner, R.B. (1994) [URE3] as an altered Ure2 protein: Evidence for a prion analog in Saccharomyces cerevisiae. Science 264, 566-569.

161. Wickner, S., M.R. Maurizi and S. Gottesman (1999) Posttranslational quality control: folding, refolding and degrading proteins. Science 286,1888-1893.

162. Wickner, R.B., K.L. Taylor, H.K. Edskes and M.L. Maddelein (2000) Prions: Portable prion domains. Curr. Biol. 10, R335-R337.

163. Wickner,R.B., K.L. Taylor, H.K. Edskes, M.L. Maddelein, H. Moriyama and B.T. Roberts (2001) Yeast prions act as genes composed of self-propagating protein amyloids. Adv. Protein Chem. 57, 313-334.

164. Wilkinson, K.D. (2000) Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. Semin. Cell Dev. Biol. 11,141-148.

165. Wyttenbach, A., O. Sauvageot, J. Carmichael, C. Diaz-Latoud, A.P. Arrigo and D.C. Rubinsztein (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. PNAS USA 97, 2898-2903.

166. Yang, S., M. Jamie, T.V. Cope and D.G. Drubin (1999) Sla2p is associated with the yeast cortical actin cytoskeleton via redundant localization signals. Mol. Biol. Cell 10, 2265-2283.

167. Zadorskii, S.P., I. Sopova and S.G. Inge-Vechtomov (2000) [Prionization of the Pichia methanolica SUP35 gene product in the yeast Saccharomyces cerevisiae] Genetika 36, 1322-1329.

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